Postmenopausal Hormone Therapy:

Differential Impact of Dose and Regimens on Hemostasis and Inflammation

by

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Abbreviations

a	activated
APC	activated protein C
APTT	activated partial thromboplastin time
AT	antithrombin
CAD	coronary artery disease
CAT	calibrated automated thrombogram
CEE	conjugated equine estrogen
CHD	coronary heart disease
CI95	95% confidence interval
CRP	C-reactive protein
CV	Coefficient of variation
CVD	cardiovascular disease
DVT	deep vein thrombosis
E2	17β-estradiol
EE2	17α-ethinylestradiol
ELISA	enzyme-linked immunosorbent assays
ER	estrogen receptor
ETP	endogenous thrombin potential
EVTET	Estrogen in Venous ThromboEmbolism Trial
EWA	Estrogen Women Atherosclerosis
F	factor
F_{1+2}	prothrombin fragments 1+2
FSH	follicle stimulating hormone
HT	hormone therapy
ICAM-1	intercellular adhesion molecule-1
INH	inhibin
LH	luteinizing hormone
Lp(a)	lipoprotein-a
MCP	monocyte chemoattractant protein
MP	micronized progesterone

MPA	medroxyprogesterone acetate
nAPCsr	normalized APC sensitivity ratio
NETA	northisterone acetate
OC	oral contraceptive
PAI-1	plasminogen activator inhibitor- 1
PAR	protease activated receptor
PC	protein C
PE	pulmonary embolism
PS	protein S
PT	prothrombin time
RCT	randomized clinical trials
SERM	selective estrogen receptor modulators
RET	Raloxifene Estrogen Tibolone
SPSS	Statistical Package for the Social Sciences
TAFI	thrombin activated fibrinolysis inhibitor
TAT	thrombin-antithrombin
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF β	transforming growth factor β
TM	thrombomodulin
TNFα	tumor necrosis factor α
tPA	tissue plasminogen activator
VT	Venous thrombosis
VWF	von Willebrand factor

Summary

Postmenopausal hormone therapy (HT) is currently mainly used for a limited time-period to relieve climacteric symptoms and for the prevention of osteoporosis. Over the last decade, reports have focused on adverse effects of HT, such as increased risk of cardiovascular disease (CVD), venous thrombosis (VT), and breast cancer. Several studies have shown that oral HT containing estrogen in conventional doses is associated with an increased risk of VT and an activation of coagulation. Moreover, it has recently been shown that even minor changes in clotting factors and coagulation inhibitors within normal ranges may account for such activation. The aim of this thesis was to compare the impact of four HT regimens on markers of activated coagulation and to identify potential mechanisms for activated coagulation by analyzing markers of vascular disease. In particular, we wanted to investigate whether lower dose of HT decreased impact on the markers.

We have found that the four regimens had markedly different impact on markers of activated coagulation, coagulation factors, coagulation inhibitors, fibrinolytic factors, and on markers of inflammation. The conventional- and low-dose HT groups generally showed similar effects, with more pronounced effects in the conventional-dose HT group. In women with previous thrombosis, oral HT containing estradiol was associated with a marked and rapid increase in CRP, whereas transdermal treatment was not. Moreover, HT appeared to reduce levels of endothelial markers both in healthy women and in women with high risk of thrombosis.

These findings are probably clinically relevant, and indicate that low-dose HT has a more favorable risk to benefit profile than conventional-dose HT. We believe that the differential effects of the regimens on coagulation and other markers reflect a differential risk of VT. Moreover, in women with high risk of thrombosis, the differential effects on CRP of oral and transdermal HT suggest that transdermal therapy is safer.

List of papers

I.

Eilertsen AL, Qvigstad E, Andersen TO, Sandvik L, Sandset PM. Conventional-dose hormone therapy (HT) and tibolone, but not low-dose HT and raloxifene, increase markers of activated coagulation. Maturitas 2006; 55: 278-87.

II.

Eilertsen AL, Sandvik L, Mowinckel MC, Andersen TO, Qvigstad E, Sandset PM. Differential effects of conventional and low dose oral hormone therapy (HT), tibolone, and raloxifene on coagulation and fibrinolysis. Thromb Res 2007; 120: 371-9.

III.

Eilertsen AL, Liestøl S, M. Mowinckel M, H. C. Hemker H.C., Sandset PM. Differential impact of conventional and low dose oral hormone therapy (HT), tibolone and raloxifene on functionality of the activated protein C system. Thromb Haemost 2007; 97: 938-43.

IV.

Eilertsen AL, Sandvik L, Steinsvik B, Sandset PM**.** Differential impact of conventional dose and low dose hormone therapy (HT), tibolone and raloxifene on C-reactive protein (CRP) and other inflammatory markers. J Thromb and Haemost 2008; 6: 928-34

V.

Eilertsen AL, Hoibraaten E, Os I, Andersen TO, Sandvik L, Sandset PM. The effects of oral and transdermal hormone replacement therapy on C-reactive protein levels and other inflammatory markers in women with high risk of thrombosis. Maturitas 2005; 52: 111-8.

1. Introduction

1.1 Menopause

The word “menopause” is derived from ***men*** and ***pausis*** and is a description of the physiological event when menstruation ceases to occur. The word “climacteric” is a Greek derivation of “the ladder” or “steps of a ladder”, and is defined as the whole period with decreasing hormone production, which usually has duration of five years on each side of the menopause. Natural menopause is commonly defined as age at the last menstrual period, which is determined retrospectively, when a woman has experienced 12 consecutive months of amenorrhoea without any obvious intervening cause. Mean age at natural menopause in Caucasian populations is between 50-52 years [1-3]. Since average age of menopause has been fairly constant over time, and women in most societies live longer, a longer period of a woman`s life is postmenopausal.

Menopause is a physiological event that occurs in all women who reach midlife, and is a complex transition involving biological, physiological, social, and cultural factors. Climacteric symptoms refer to an increase of vasomotor symptoms, such as “hot flushes” and night sweats, insomnia, and urogenital atrophy [4-7]. Many other symptoms, i.e., irregular menstrual bleeding, osteoporosis, cardiovascular disease (CVD), depressed mood, and irritability are associated with menopause, but are not necessarily correlated to estrogen levels.

1.1.2 Changes in hormones

In the fertile period, almost all active estrogen, i.e., 17β–estradiol (E2), is produced by granulosa and theca cells in ovaries and their luteinized derivates [8]. During menstrual cycles, the production of E2 varies cyclically and reaches peaking levels just before ovulation. After the follicle ruptures at ovulation, the corpus luteum secretes progesterone, but small amounts are also produced by the adrenal glands. The cyclical pattern is regulated by positive and negative feedback mechanisms, i.e., complex interactions between hormones from the hypothalamus, the pituitary and the ovaries. During the follicular phase of the cycle, levels of E2 stimulate secretion of gonadotrophins, while progesterone and inhibin B (INH-B) contributes to control of luteinizing hormone (LH) and follicle stimulating hormone

(FSH) secretion, respectively. In the luteal phase both E2 and progesterone regulate the gonadotrophins, while inhibin A (INH-A) are involved in the FSH secretion [9].

The progressive loss of follicles of the ovaries leads to an absence of follicular function and a permanent cessation of menstruation. The serum patterns of ovarian and pituitary hormones alter as the menopause approaches, and a decreased secretion of INH-B from the granulosa-cells leads to an initial slow rise in FSH. As the follicle number progressively decreases, the reduced levels of INH-B and E2 lead to a more marked increase in the gonadotrophins, increased level of FSH and a later rise in LH [10].

Estrone (E1), which is mainly produced in the adrenal cortex, is the dominant estrogen in postmenopausal women. Moreover, levels of progesterone and E2 are very low and most of the E2 is formed by extragonadal conversion from androstendion and testosterone [8].

1.2 Postmenopausal hormone therapy

1.2.1 History

Various hormone preparations have been used in the treatment of climacteric symptoms since the end of the 19th century, and different estrogen formulations have for more than 60 years been approved by the US Food and Drug Administration as such treatment [11]. HT had increasing popularity for decades, until studies in the mid 1970s revealed that treatment with estrogen alone was associated with an increased risk of uterine cancer [12;13]. It has later been shown that by combining estrogen with a progestin, the increased risk of endometrial hyperplasia and uterine cancer was avoided [14;15]. It is therefore recommended that estrogen to be given in combination with a progestin in women with a preserved uterus. The increasing use of HT in the 1980s was probably related to publication of reports showing effective protection of estrogen in preventing bone-loss [16]. Epidemiological studies showing reduced risk of CVD in estrogen-users further increased the popularity in the 1990s [17;18]. The large randomized clinical trials (RCT): Heart Estrogen/progestin Replacement Study (HERS) and Women's Health Initiative (WHI) have reported potential greater harm than benefit of combined estrogen/progestin treatment in conventional doses, both in healthy women and in women with vascular disease [19;20]. Moreover, the Estrogen in Venous Thromboembolism Trial (EVTET) revealed an increased risk of recurrent VT by use of HT in women with a history of VT [21]. These findings altered

the indication for use in clinical practice and caused a profound decrease in the use of HT. Currently, HT is mostly used for a limited period to relieve climacteric symptoms, and for the prevention of osteoporosis.

HT was initially administered as oral estrogen, but since the 1980s new forms of application such as patches, gels, nasal spray, vaginal rings, and vaginal tablets became available [11]. Various formulations of estrogens, progestins, and estrogen-progestin combinations have been approved for treatment of climacteric symptoms. HT can be administered continuously, with a fixed daily dose of estrogen and progestin, or with intermittent administration of a progestin. In perimenopausal women, intermittent administration of progestin is preferable in order to regulate genital bleeding.

Tibolone is a synthetic steroid compound with estrogenic, progestogenic and androgenic properties. It has been used in Europe since the beginning of the 1980ies, and the indications for use are similar to conventional HT: prevention of postmenopausal osteoporosis and treatment of climacteric symptoms [22].

Selective estrogen receptor modulators (SERMs) have also been available for decades. The interest in the SERMs increased after recognition of their selective agonist and antagonist effects on various estrogen target tissues. Tamoxifene was developed as an antiestrogen for the treatment of breast cancer. It maintains bone density, but stimulates the endometrium [23] which implies an increased risk of endometrial cancer [24]. Raloxifene is an alternative to estrogen in prevention and treatment of osteoporosis.

1.2.2 Estrogens

The naturally occurring estrogens; E2, E1 and estriol (E3), are C_{18} steroid hormones derived from cholesterol. In addition to the naturally occurring estrogens, wide ranges of natural and synthetic substances possess estrogenic activity. Estrogens have multiple biological effects that are determined by the structure of the hormone, the subtype of the estrogen receptor (ER) involved, and the tissue distribution of the receptors. The different estrogen ligands have differential affinity for the ER [8]. Many types of estrogens and estrogen-derivatives are utilized in postmenopausal HT and oral contraceptives (OC). Conjugated equine estrogens (CEE) are most commonly used in the US and were tested in the WHI and HERS studies. In the Northern Europe E2 is mainly used. The different types of estrogens are shown in table 1.

1.2.3 Progestagens (progestogens or gestagens)

Progestagens are steroid hormones that produce effects similar to those of progesterone, the only natural progestagen. Progesterone is the most potent progestagen. The function of progesterone in the fertile woman is to prepare the genital tract for reception and maturation of the fertilized ovum and to maintain pregnancy. All other progestagens are synthetic and are often referred to as progestins. Progestagens differ in affinity for the progesterone receptor and have androgenic, antiandrogenic, anti-mineralocorticoid, estrogenic and anti-estrogenic properties. Progestins can be classified according to source of the derivative; 17-α–hydroxyprogesterone (structural similarities with progesterone) and 19-nortestosterone (structural similarities with testosterone) [25]. Norethisterone acetate (NETA) is the most commonly used progestin in HT in Northern Europe, while medroxyprogesterone acetate (MPA) and micronized progesterone (MP) are most commonly used in the US. The different progestagens are shown in table 1.

ESTROGENS		**PROGESTAGENS**	
Natural	**Synthetic**	**17-α-OH progesterone**	**19-nortestosterones**
HUMAN	**STEROIDS**	**PREGNANES**	**ESTRANES**
Estradiol	Etinyl estradiol	Medroxyprogesteronacetate	Norethisterone
Estriol	Mestranol	Megestrole acetate	Norethisterone acetate
Esterone		Cyproterone acetate	Lynestrenole
		Dydrogesterone	
ESTERS	**NON-STEROIDS**		**GONANES**
Estradiol-valerate	Dienestrole		Norgestrel
Estrone sulphate			Levonorgestrel
			Desogestrel
			Gestodene
CONJUGATED	**OTHER**		Norgestimate
Sodium estrone-sulphate	Tibolone		
Sodium equiline-sulphate			
Sodium equileine-sulphate			

Table 1 Types of Estrogens and Progestagens

1.2.4 Tibolone

Tibolone has been classified as a selective tissue estrogenic activity regulator (STEAR) [26]. It is structurally related to 19-nortestosterone derivatives and is after oral administration converted into three primary active agents: 3-α-hydroxy-tibolone, 3-β-hydroxy-tibolone and Δ-4-tibolone [27]. The specific effects of tibolone in different tissues depend on the presence

of one of its active metabolite and its binding and activation of the associated receptor. The hydroxyl-tibolone metabolites exert estrogenic effects on the bone, vagina, and climacteric symptoms. The progestogenic activities of the Δ–4-isomer prevent stimulation of the endometrium, whilst the androgenic activities may play a role in the liver and brain. RCTs have shown beneficial effects of tibolone on prevention of bone loss, and tibolone is associated with a low incidence of vaginal bleeding [22].

1.2.5 Raloxifene

SERMs are a group of compounds that is structurally related, but chemically different from estrogen. They lack steroidal structure, but possess a tertiary structure that allows them to bind to the ER. SERMs exert selective estrogen agonistic or antagonistic effects, depending on the target tissue [23].

Raloxifene hydrochloride is a benzothiophene that binds to ER, and is classified as a SERM. In postmenopausal women, raloxifene increases bone mineral density and reduces the risk of vertebral fractures [28]. It is approved for use in prevention and treatment of osteoporosis in several countries, whilst it is not indicated for the treatment of climacteric symptoms due to an anti-estrogenic effect in hypothalamus. Raloxifene has estrogen-agonist effects on lipoproteins and lipid metabolism, whereas estrogen antagonistic effects are seen in breast and uterus. It is associated with a reduced risk of breast cancer in osteoporosis prevention trials [29;30] and in women with increased risk of CVD [31].

1.3 Hemostasis

1.3.1 Normal hemostasis

Hemostasis describes the biological process responsible for maintaining proper blood flow and the physiological cessation of bleeding after vascular injury. The process involves the vessel wall, platelets, and plasma-proteins. At the site of tissue damage, platelets circulating in the blood rapidly adhere to collagen and other extracellular matrix components of the subendothelium. The platelets subsequently recruit additional platelets and leukocytes to form the platelet plug. The coagulation system is activated by tissue factor (TF), which is exposed to circulating blood either by vascular damage or by production and exposure on the surface of blood monocytes. When coagulation is initiated, it proceeds through a series of activation steps that ends in the generation of thrombin. Thrombin cleaves fibrinogen to

generate fibrin. Concomitant activation of naturally occurring anticoagulants and the fibrinolytic system are important regulatory mechanisms of clot formation. This delicate balance can be disrupted resulting in thrombosis or abnormal bleeding [32].

Over the last century, several theories for understanding the underlying mechanisms of hemostasis have been proposed. The biochemical era of blood coagulation began with the theories of Schmidt and Morawitz who suggested that activation of prothrombin to thrombin, by thromboplastins (later named TF), and conversion of fibrinogen to fibrin by thrombin, were important reactions involved in clot formation. The original description of the coagulation process as a "cascade" or "waterfall" reaction was based on circumstantial evidence and the discovery of coagulation proteins in the mid 20th century [33]. These models have later been modified. In a later version, the interactions between the coagulation proteins was outlined in two distinct activation pathways; the extrinsic (TF-induced) pathway and intrinsic (contact activation) pathway converging on a "common" pathway, with thrombin generation as the end-point of the reactions. The components of the extrinsic pathway are measured in the prothrombin time (PT) assay, whilst the factors in the intrinsic pathway are measured in the activated partial thromboplastin time (APTT) assay. Although these models have been useful in clinical coagulation laboratory tests, they have been shown to be insufficient in explaining the connection between coagulation factor deficiencies and clinical phenotypes (e.g., the serious bleeding tendency in patients deficient of FVIII or FIX although the extrinsic pathway was intact). Models that are more recent reflect the coagulation process *in vivo*, involving the vessel wall, platelets, and plasma-proteins and are consistent with clinical conditions. Our contemporary understanding of hemostasis is that coagulation occurs on specific cell surfaces in distinct, but overlapping steps; initiation, amplification and propagation [34;35]. The coagulation process starts on TF-bearing cells and proceeds on the surface of activated platelets.

1.3.2 Cell based model of coagulation

The ***initiation phase*** is triggered when TF is exposed to blood. TF is a transmebrane glycoprotein that is constitutively expressed in cells of the subendothelium or extravascular tissues and is exposed to plasma upon vascular injury. Moreover, synthesis of TF is inducible in monocytes and other cells by a number of stimuli and may be expressed in microparticles from different origins. The circulating factor (F)VII/FVIIa in plasma binds to TF forming the

TF/FVII(a) complex (figure 1). The activated TF/FVIIa complex cleaves zymogen forms of FIX and FX to their active enzyme forms (FIXa and FXa). In plasma, the serine protease inhibitors antithrombin (AT) and TF pathway inhibitor (TFPI) rapidly inactivate FXa, whereas FXa localized on a phospolipid membrane surface, e.g., in proximity of TF, is relatively protected from inactivation. FIXa is more stable and can dissociate from the TF-bearing phospholipid environment and dock on a nearby, activated platelet or another cell surface. FIXa is not inhibited by TFPI and are only slowly inactivated by AT. FXa that remains on the TF-phospholipid surface can associate with FVa to form the prothrombinase complex to generate small amounts of thrombin. FVa derives from several sources; circulating FV can be activated by thrombin or FXa, or secreted from the activated platelet.

The small amounts of thrombin produced in the initiation phase have several important functions in the ***amplification phase.*** Low concentration of thrombin fully activates platelets adhering to the site of injury. On the surface of the platelet, thrombin activates FV, FXI, and FVIII by cleaving it from von Willebrand factor (VWF).

In the ***propagation phase,*** procoagulant complexes assemble on the surface of the activated platelets to accelerate thrombin generation. FIXa binds to FVIIIa on the surface of the activated platelets to form the FIXa/VIIIa complex (*tenase*), which converts FX to FXa. Moreover, platelet-bound FXIa can activate FIX to form more tenase. FXa then rapidly associates with FVa to form the *prothrombinase-complex*, which cleaves prothrombin to thrombin and prothrombin fragments 1+2 (F_{1+2}) on the platelet surface. F_{1+2} is thereby a marker of thrombin generation (figure 2). Thrombin then catalyzes the conversion of fibrinogen to form soluble fibrin monomers, which spontaneously polymerizes into non-covalently insoluble fibrin-polymers. Finally, the fibrin-clot is stabilized by cross-linking by FXIIIa, which is activated by thrombin (figure 2).

Thrombin is involved in the regulation of proinflammatory processes including the expression of leukocyte adhesion molecules on endothelial cells, leukocyte chemotaxis, activation of platelets, and it has properties as a growth factor [36].

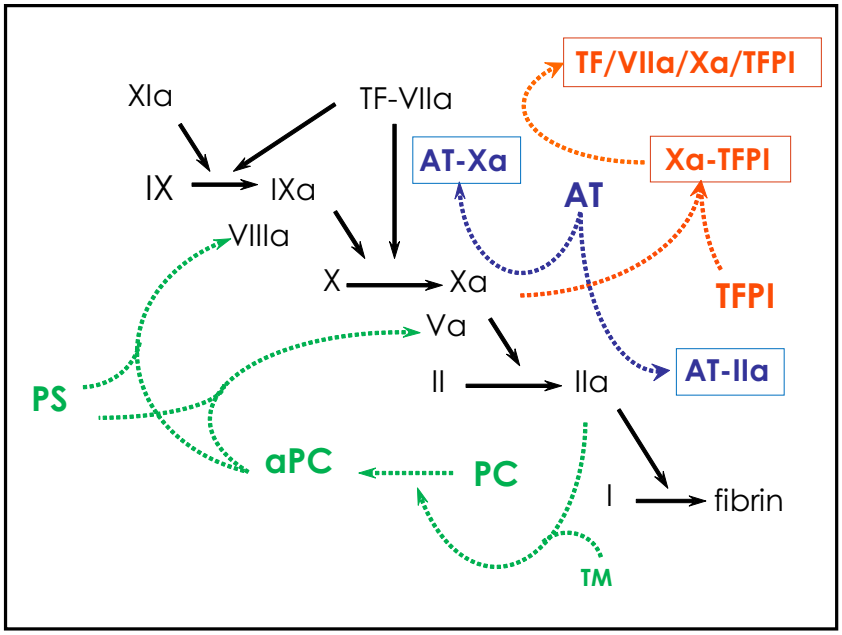

Figure 1 Schematic overview of the regulation of coagulation

AT is the main inhibitor of thrombin, but also inactivates FXa, FIXa, FXIa (blue color), and FVIIa bound to TF. TFPI inhibits both FXa and the TF/VIIa complex. Initially TFPI binds to FXa, and then the TFPI/FXa complex binds to the TF/FVIIa complex forming a quaternary TFPI/FXa/TF/FVIIa complex (red color). The PC system exerts its anticoagulant effect by regulating the activity of FVIIIa and FVa, which function in the *tenase* and the *prothrombinase-complex*, respectively (green color).

1.3.3 Regulation of blood coagulation

Several naturally occurring anticoagulant pathways regulate the activity of the coagulation system. The most important pathways include a series of anticoagulant proteins and cofactors which bind to activated coagulation factors and down-regulate their activity [37].

AT is a serine protease inhibitor that, in addition to be the important inhibitor of thrombin, can inhibit activated coagulation serine proteases, including FIXa, FXa, and FXIa (figure 1), and also FVIIa bound to TF. The inhibitory activity of AT is accelerated by specific interactions with the cofactors heparin and heparan sulphate proteoglycans. The heparan sulphate on the endothelium and subendothelium localize AT activity to the vessel wall and maintain its natural non-thrombogenic properties [38]. AT has also been shown to have anti-inflammatory functions, probably mediated by signaling through heparan sulphate on

endothelial and leukocyte cell surfaces. Thrombin bound to AT, i.e. TAT-complex, is both a marker of generated thrombin and its inactivation by AT (figure 2).

TFPI, the physiological inhibitor of TF, is mainly produced in endothelial cells and platelets. TFPI is found in different forms and is localized in association with endothelial cells, and platelets. It is present in the blood in a free form or bound to lipoproteins. TFPI inhibits both FXa and the TF-VIIa complex. Initially, TFPI binds to FXa, and then the TFPI/FXa complex binds to the TF/FVIIa complex forming a quaternary TFPI/FXa/TF/FVIIa complex [39]

The protein C (PC) anticoagulant pathway is a major regulatory mechanism that is activated by the thrombin-thrombomodulin (TM) complex [40]. Activation of PC is initiated by the binding of thrombin to TM and endothelial protein C receptor (EPCR) on the endothelial surface and this complex activates PC. When activated PC (APC) dissociates from the EPCR, it interacts with its cofactor protein S (PS) on membrane surfaces to catalyze the inactivation of FVa and FVIIIa, the important cofactors of the *tenase* and the *prothrombinase-complexes* (figure 1) [41].

APC resistance is a phenomenon characterized by poor response to APC, i.e., low sensitivity to APC, when APC is added to plasma *ex vivo*. Inherited APC-resistance is caused by the FV_{Leiden} mutation, which is due to a specific point mutation in exon 10 (G1691A shift) of the FV gene. The mutation predicts an arginine to glutamine substitution in position 506 of the mature FV molecule. Since the 506 position of the FV molecule is one of three sites at which APC cleaves and inactivates FVa, the mutation delays inactivation of FVa [42]. An acquired form of APC resistance may be seen in certain conditions, such as pregnancy or exogenous hormone therapy, i.e., OC and postmenopausal HT use [43;44]. APC resistance can be measured in plasma using an APTT based assay, but more recently new methods based on thrombin generation, expressed as normalized APC sensitivity ratios (nAPC-SR) have been developed (see Methods and Discussion) [45].

1.3.4 Fibrinolysis

The fibrinolytic system is a series of enzymes responsible for the proteolytic elimination of fibrin. The activity of the fibrinolytic system limits clot formation and determines the persistence of the thrombi in the vascular system.

Plasmin, the key enzyme of fibrinolysis, degrades the fibrin clot into fibrin-fragments known as fibrin-degradation products (FDP). D-dimer is a specific FDP that contains the

cross-linked region of fibrin. D-dimer is thus a marker of fibrin formation and subsequent degradation of fibrin by plasmin. Moreover, increased D-dimer is a marker of activated coagulation and ongoing fibrinolysis (figure 2). Plasmin is formed by activation of the circulating inactive zymogen plasminogen. The main activator is tissue plasminogen activator (tPA), a serine protease synthesized, stored, and released from endothelial cells. Urokinase plasminogen activator (uPA) can also activate plasminogen. These plasminogen activators are inhibited by plasminogen activator inhibitor-1 (PAI-1). PAI-1 is produced in many cell lines, including endothelial cells, and release of PAI-1 can by stimulated by many different compounds, including cytokines, thrombin, and endotoxins [46]. Thrombin, when bound to TM, can activate thrombin activable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase B. TAFI inhibits fibrinolysis through several mechanisms. Activated TAFI (TAFIa, carboxypeptidase B) removes N-terminal lysine residues of fibrin. These lysine residues are important binding-sites for tPA and plasmin(ogen). TAFIa thereby prevents fibrinolysis and enhances clot resistance [47].

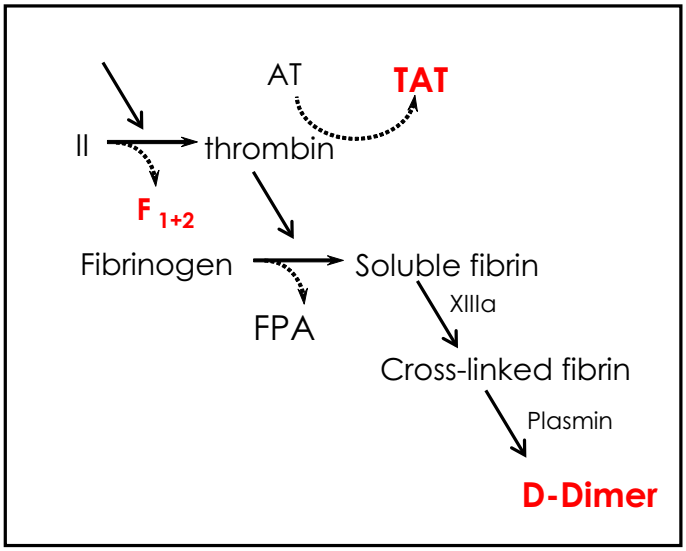

Figure 2 Markers of activated coagulation

D-dimer is a marker of fibrin formation and consequent degradation of fibrin by plasmin. Increased D-dimer is thus a marker of activated coagulation and ongoing fibrinolysis. F_{1+2} is formed by the cleavage of prothrombin and is a marker of thrombin generation, whereas thrombin bound to AT forming TAT-complexes is both a marker of generated thrombin and its inactivation by AT.

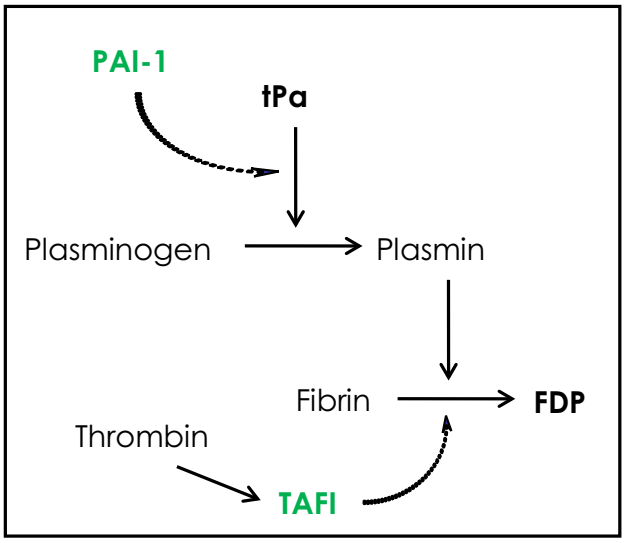

Figure 3 Schematic overview of the fibrinolysis

Plasmin degrades fibrin into fibrin-degradation products (FDP). D-dimers are specific crosslinked FDPs and specific for fibrin. Fibrinolysis is activated by tissue plasminogen activator (tPA), which cleaves plasminogen to plasmin. Fibrinolysis is inhibited by inhibitors, including plasminogen activator inhibitor-1 (PAI-1). Thrombin, when bound to thrombomodulin (TM), can activate thrombin-activable fibrinolysis inhibitor (TAFI), which can inhibit fibrinolysis through different mechanisms.

1.4 Endothelial functions and dysfunction

Research during the last decades has revealed that the vascular endothelial cells by secretion or surface expression of a series of specific molecules are involved in maintaining vascular homeostasis. Under normal conditions the endothelium prevents leukocyte and platelet adhesion to the vascular wall, displays anticoagulant and fibrinolytic properties, inhibits proliferation of vascular smooth muscle cells, and controls vasoconstriction. Localized alteration or loss in these functions, induced by a variety of stimuli, lead to endothelial dysfunction, increasing the adhesiveness of the endothelium i.e. leukocyte adherence and platelet activation. The injury also induces the endothelium to have increased permeability and increased production of cytokines and growth factors [48-50].

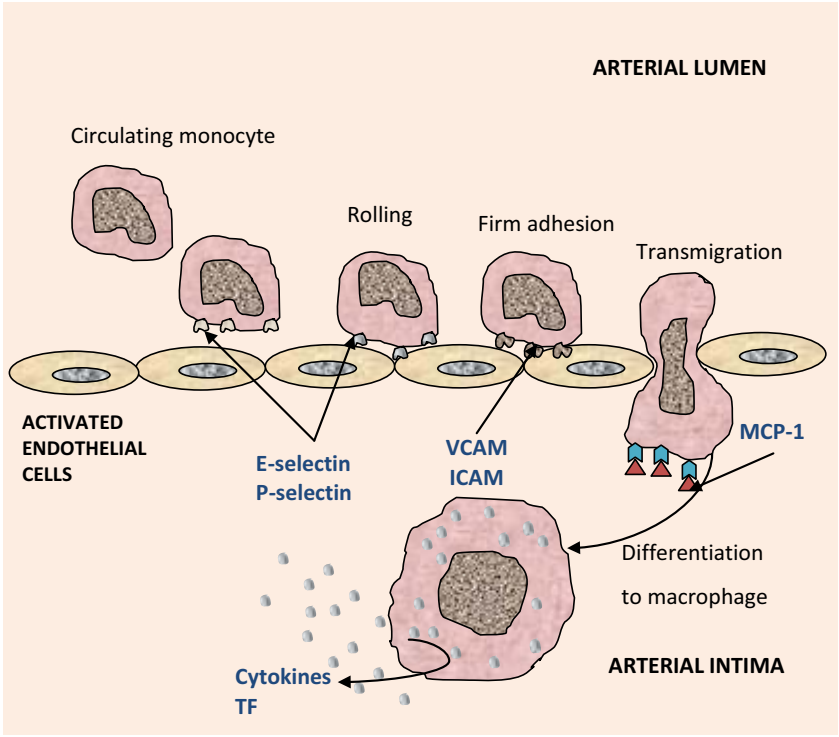

Figure 4 Leukocyte-endothelial cell interactions on activated endothelial cells.
Activated endothelial cells express adhesion molecules and chemokines that facilitate activation, adhesion, transmigration and differentiation of circulating leukocytes (mainly monocytes and T-cells) in the subintimal space. Selectins and their ligands are involved in the initial leukocyte rolling and tethering of leukocytes on the vascular wall. Integrins (VCAM and ICAM) induce firm adhesion at the endothelial cell surface. MCP-1 and other chemokines participate in the process of migration to the subintimal space, where the monocytes differentiate into macrophages.

1.4.2 Cell adhesion molecules (CAMs) and endothelial and inflammatory markers

CAMs, like intercellular adhesion molecule-1 (ICAM-1), E-selectin, and P-selectin, are expressed on activated endothelial cells as a response to vascular injury [48;49]. CAMs are induced by proinflammatory cytokines, like interleukin 6 (IL-6) and tumor necrosis factor α (TNFα), and facilitate leukocyte attachment to endothelial cells and migration into subintimal space of the arteries during vascular inflammation (figure 4). Monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL-2), controls transendothelial migration and differentiation of monocytes into macrophages in subendothelial space and thereby mediates the recruitment of macrophages to the arterial

lesion [51]. TM is responsible for thrombin-induced activation of PC on the endothelial surface, and has been shown to suppress endothelial cell responses to inflammatory mediators. Moreover, expression of TM is reduced in endothelium overlying atherosclerotic plaques [52]. Increased levels of TM have been reported in various clinical conditions as CVD, diabetes, and endothelial injuries [53]. Enhanced secretion of VWF in response to inflammatory secretion has been shown to increase platelets interaction with endothelial cells [54].

C-reactive protein (CRP) has been considered to be a non-specific acute phase marker of tissue injury, infection or inflammation. Several studies have shown that CRP, in levels within normal range and as a marker of low-grade systemic inflammation, is a useful predictor of coronary artery disease (CAD) [55-57]. Whether CRP is a marker of atherosclerotic burden, or is directly involved in atherosclerosis is under investigation [58]. Moreover, it is unclear whether the relationship between CRP and inflammation is independent of other inflammatory markers.

1.5 Venous thrombosis, epidemiology and risk factors

The annual incidence of VT is 1 to 3 individuals per 1000 per year in a general population [59;60]. The major manifestations, deep vein thrombosis (DVT) and pulmonary embolism (PE) are associated with considerable acute and chronic morbidity. Acute death of PE occurs in 1-2 % of the patients, while post-thrombotic syndrome is a disabling sequele after DVT occurring in 15% to 50% of the patients [61]. More rarely thrombosis occurs in other veins, such as cerebral sinus veins, portal and liver veins, and upper extremity veins. Nevertheless, the diagnosis of VT can be difficult and remains a challenging clinical problem. The incidence of VT increases exponentially with age from approximately 1 per 100 000 in childhood, and increases to 1 per 100 for those over the age of 75 years. The annual incidence of VT was 0.71 per 1000 in females between 40-59 years in a French study [59].

In the mid 19th century, Virchow postulated three major causes of thrombosis: changes in the vessel wall, alterations in blood flow, and changes in blood composition (hypercoagulability) [62]. This understanding of pathogenic factors is known as Virchow`s triad and is still considered useful to illustrate the pathogenesis of thrombosis. The biochemical and molecular basis of these components are progressively unraveled.

1.5.2 Inherited and acquired risk factors for venous thrombosis

VT is considered to be a multicausal disease. Inherited and acquired risk factors may increase the risk of VT either in an additive or a synergistic fashion, and thereby contribute to the development of VT [63;64]. Many of the inherited risk factors for VT known today have a high prevalence in the general population. Many of the acquired risk factors are also common. A summary of known risk factors for VT is shown in table 2.

ACQUIRED	INHERITED	MIXED/UNKNOWN
Age	Antithrombin deficiency	High levels of factor VIII,
Previous thrombosis	Protein C deficiency	High levels of factor IX
Immobilization	Protein S deficiency	High levels of factor XI
Trauma	Factor V Leiden mutation (FVL)	High levels of fibrinogen
Plaster cast	Prothrombin gene mutation	High levels of TAFI
Major surgery		Low levels of TFPI
Orthopedic surgery		APC resistance in absence of FVL
Cancer		Hyperhomocysteinemia
Medical disorders		
Antiphospholipid syndrome		
Oral contraceptives		
Hormone therapy		
Pregnancy		
Puerperium		
Overweight		

Table 2 Risk factors for venous thrombosis

1.5.3 Hemostatic factors and risk of venous thrombosis

Hereditary deficiencies of coagulation inhibitors, e.g., such as low levels of AT, PC, and PS are well established risk factors for VT [43;63;65]. Recently, low levels of TFPI was also shown to be associated with an increased risk of VT [66].

Factor V_{Leiden} mutation is a major risk factor for VT, and is the most common cause of APC resistance [67]. However, an APC resistant phenotype is also seen in acquired conditions and with other genetic polymorphisms [42]. APC resistance, in the absence of the factor V_{Leiden} mutation, is probably an independent risk factor for VT [68]. Several studies indicate that acquired APC resistance may be an important mechanism for the increased risk of VT associated with use of OC [43;44] and HT [69].

There is evidence that elevated levels of procoagulant factors, such as FVIII, VWF, FIX, FX and FXI, exceeding the 90^{th} percentile of the distribution of the clotting factors in the general population, are related to increased risk of VT. Studies on fibrinogen have been

inconclusive [65;70]. Moreover, increased levels of TAFI are associated with increased risk of VT [71], whilst this relation is uncertain for other fibrinolytic factors such as t-PA and PAI-I.

1.6 Atherothrombosis, epidemiology and risk factors

In developed countries CVD is the leading cause of morbidity and mortality for both men and women [72]. CAD, cerebrovascular disease and peripheral vascular disease are clinical complications of atherosclerosis, their common underlying pathophysiological process. These clinical complications are often triggered by arterial thrombosis, and the term atherothrombosis was introduced to integrate the chronic and acute aspects of the disease.

Epidemiological studies have identified risk factors that are predictive of high incidence of disease [73;74]. Predisposing factors and factors associated with increased risk are shown in table 3. Clinical manifestation of CAD in women appears about ten years later than in men, but the impact of age is more important in women and they seem to have a higher case-fatality risk of CAD [72]. The majority of classical risk factors show no important gender specific differences, although there is evidence suggesting that hypertriglyceridemia, cigarette smoking, and diabetes mellitus are associated with higher relative risk in women than in men [74]. Furthermore, there are studies suggesting that some women tend to accumulate more risk factors and therefore end up with a higher relative risk than men at same age [75].

1.6.2 Atherosclerosis and inflammation

It is now widely accepted that inflammation is involved in development and progression of atherosclerosis [49-51]. This is supported by biological data and by clinical studies showing correlations between circulating markers of inflammation and CVD. It is established that moderately elevated levels of high sensitivity CRP have been shown to be an independent risk factor of CAD [55-57]. In patients with myocardial infarction (MI), several studies have found that elevated levels of CAMs and selectins are associated with poorer outcome [48]. Moreover, levels of CAMs appear to be related to risk of CAD in healthy individuals [48]. Increased levels of TM have been reported in various clinical conditions, such as CVD, diabetes, and endothelial injuries [53]. Finally, studies have shown that increased concentrations of lipoprotein(a) (Lp(a)) are associated with higher risk of CAD [76].

Risk factors (considered to be causally linked)	**Risk markers**
Smoking	Elevated prothrombotic factors; fibrinogen, PAI-1
Elevated LDLs	Markers of inflammation
Low HDL	Elevated homocystein
High blood pressure	Elevated lipoprotein (a)
Elevated glucose	Low socioeconomic status
Physical inactivity	Physiological factors
Obesity	
Diet	

Table 3 Risk factors and risk markers for cardiovascular disease

1.7 Hormone therapy and risk of venous thrombosis

Recent epidemiological studies and RCTs have shown that HT containing estrogen in conventional doses, e.g., 2 mg E2 or 0.625 mg CEE, is associated with an increased risk for VT [19-21;77-81], both in healthy women and in women with a history of VT [21]. The risk of VT is probably higher during the first year of use [21;79], and possibly more pronounced for users of combined estrogen-progestin HT than estrogen monotherapy [82;83]. Transdermal therapy does not seem to be associated with increased risk of VT [84;85]. A recent report showed no increased risk of VT in younger women receiving HT containing E2 [86]. Epidemiological studies have failed to detect significant differences in risk of VT between various estrogen doses [80;81;87], however these studies were not powered to detect such differences. One RCT has reported no excess risk of VT on low-dose E2 in a stroke prevention trial [88]. Only one case-control study has provided information on risk of VT on tibolone treatment [87], but the numbers of users were low. Data from RCTs are lacking. Raloxifene has been found to be associated with an increased risk of VT both in healthy women, and women with increased risk of CVD [31;89].

1.8 Hormone therapy and risk of atherothrombosis

Until the early 1990ies epidemiological studies suggested a cardioprotective effect of postmenopausal HT [17;18;90;91]. More recent RCTs reported that HT was associated with a modest, but significant increase in risk for CVD [19;20]. On the other hand, estrogen monotherapy is not associated with increased risk [83]. The discrepancies between these studies are under debate, and numerous hypotheses have been put forward to explain these contradictory results [92]. Lower risk of arterial thrombosis at lower doses of HT are

suggested by one RCT [88] and two epidemiological studies [91;93]. Grodstein et al have recently published observational data supporting lower risk for CVD in users of HT in younger women starting treatment near age of menopause [94]. Their results were similar across various doses of estrogen, and among women with several CVD risk factors. The risk of atherothrombosis by use of tibolone has been poorly evaluated, whereas studies on raloxifene have demonstrated conflicting results [31;95].

1.9 Menopause and changes in hemostatic factors

Menopause is associated with minor changes in several coagulation factors, including minor increases in coagulation FVII, FVIII, and fibrinogen [96-103]. Minor increases in the coagulation inhibitors AT [96;104], PC [105], PS [106], and TFPI [66] are observed in healthy postmenopausal women, whereas markers of activated coagulation, e.g., F_{1+2} and TAT-complexes seem to be unaffected by menopausal status [107]. Significant changes in the fibrinolytic factors including higher levels of PAI-1, and tPA are observed in postmenopausal women [103;108].

Studies have reported that menopause induces alterations in the cytokine profile. Reduced levels of transforming growth factor β (TGFβ) have been detected [109], but studies on TNFα and IL-6 have shown conflicting results [109;110]. Levels of CAM and P-selectin have been shown to be higher in postmenopausal women, as compared to premenopausal women [111], while levels of CRP have appeared to be unaffected by menopausal status [103;110].

1.10 Hormone therapy and effects on coagulation

Several studies have evaluated the effects of HT on coagulation. The effects on markers of activated coagulation, on selected coagulation factors, coagulation inhibitors and markers of fibrinolysis are presented in table 4, 5 and 6. Only studies with a randomized design are included. Studies published both prior to (blue color) and later than the RET-study are included.

1.10.1 Markers of activated coagulation

There is now compelling evidence that oral HT containing conventional dose of estrogen is associated with increased levels of F_{1+2} [112-118]. Studies investigating the effects on D-

dimer [114-121] and TAT complexes [113;116;118;122] have shown conflicting results. Two studies have demonstrated increased levels of D-dimer after 12 weeks of treatment with tibolone, but these changes did not sustain after 6 months [123;124]. Studies investigating the effect of tibolone on F_{1+2} are diverging [123;125;126]. Studies evaluating raloxifene have shown no significant changes in D-dimer, F_{1+2} and TAT complexes [127;128].

1.10.2 Coagulation factors

Most studies using oral estrogen alone have reported increase in levels of FVII [113;116;117;129-131], whereas studies evaluating continuous, conventional-dose, combined HT have shown no effect on FVII [114;116;132], suggesting that progestin modifies the effects on FVII. Studies on E2 and progestin have even shown reduced levels of FVII [118;132]. Studies on FVIII and VWF have shown no change [113;114;117;118;130;133-135]. There are few reports on other coagulation factors.

RCTs on HT have reported inconsistent results on fibrinogen [113;114;117-119;129;130;132;136-140], as opposed to RCTs on raloxifene which consistently have demonstrated decreased levels [127;141;142]. There are also few studies evaluating the effects of tibolone on procoagulant factors, but there is evidence for reduction in FVII, and no effect on FVIII [123;124;143]. Studies on the effects of tibolone on fibrinogen have been inconclusive [123;124;126;143].

1.10.3 Coagulation inhibitors

Oral HT affects all known pathways of coagulation inhibition. AT is lowered by 5-10% [112-114;116;118;129;130;132;144], PC by 5-15% [116;118;132;144], PS by 0-5% [112;144] and TFPI by 10-30% [118;122;138;145;146]. There are limited data on the effects of tibolone. Some studies have found reduced levels of AT [124;125]. Studies on PC and PS are discordant [123;147]. Studies evaluating the effects of raloxifene on coagulation inhibitors have shown reduction in AT [128;148-150], whereas the effects on PC and PS have been poorly evaluated. No clinical studies evaluating the effects of raloxifene on TFPI are yet available.

Recent studies suggest that HT, both conventional and lower doses, may reduce sensitivity to APC [69;134;151;152]. Treatment with raloxifene does not seem to alter the

sensitivity to APC [128;153], and there is only one study evaluating the effects of tibolone [123].

1.10.4 Fibrinolysis

Several studies have reported evidence of increased fibrinolytic activity by use of HT, with decreased levels of PAI-1 [114-117;154-156], and increased levels of tPA [128]. In contrast to the other hemostatic markers, have the effects of tibolone on markers of fibrinolysis (e.g., PAI-1 and t-PA) been more extensively examined. Most studies have reported lowered PAI-1 levels [123;124;126;157], and with a parallel decrease in t-PA [127;128;142]. OC have recently been shown to increase TAFI. The effects of HT is not clear, but one report found that HT decreased TAFI in women homozygous for the rare TAFI -438 A allele [71]. The effects of tibolone on TAFI levels have so far not been investigated. Studies on raloxifene have shown no effect on PAI-1 [127;128;142;158].

1.11 Hormone therapy and effects on endothelium and inflammation

Several studies on postmenopausal HT in conventional doses, i.e., 2 mg E2 or 0.625 mg CEE have reported marked increase in CRP [135;138;155;159-164]. The increased CRP seems to be independent of elevated levels of IL-6 [160;162-165]. The effects of different progestins on CRP levels have been contradictory. The PEPI-trial showed no difference in CRP between different progestin components [135], whereas other studies have suggested that progestin attenuates the increase in CRP in a dose dependent manner [164]. Oral HT has been shown to reduce levels of ICAM, P-selectin, E-selectin, MCP-1 and Lp(a)[139;160;165-173]. Interestingly, some studies suggest that regimens with lower doses of HT, both with CEE and E2, to a lesser extent increased the CRP levels [174-176], whereas the favorable effects on endothelial markers were preserved. Transdermal treatment appears to have minor impact on markers of inflammation, as most studies both with or without different progestins have found no effect on CRP [117;177-179] and only minor lowering effects on CAMs [117;180].

Studies evaluating the effect of tibolone on CRP levels have been conflicting [125;181-184], but several reports have indicated that CRP levels are unaffected by raloxifene treatment [127;169;185;186].

Author	Design	Treatment	N	DD	F 1+2	TAT	AT	PS	PC	TFPI	APC-R	FVIIa	FVIIag	FVIII	Fg	PAI-1
Lindberg 1989, [129]	Open, CO 12 weeks	Ethinyl estradiol (EE) 10 µg Estradiol valerate (E_2V) 2mg	24				↓ ↓						↑ →	↑ →	→ →	
Sporrong 1990, [132]	RCT(B) 12 months	ccE2 2mg+1mg NETA ccE2 2mg+0.5mg NETA ccE2 2mg +megestrolacetat 5mg ccE2 2mg +megestrolacetat 2.5mg	60				↓ → → →		↓ → → →			↓ ↓ → →	↓ ↓ → ↑		→ → → →	→ → → →
Caine 1992, [112]	RCT,CO 3 months	CEE 0.625mg CEE 1.25mg Placebo	29		↑ ↑		↓ ↓	↓ ↓	→ ↑							
Kroon 1994, [113]	Open, CO 12 weeks	CEE 0.625 mg td E2 50µg/24 t	23		↑ ↑	↓ →	↓ →	→ →	→ →			↑ ↑	↑ ↑	→ →	→ →	↓ →
PEPI 1995, [135;137]	RCT (B) 3 years	CEE 0.625mg CEE 0.625mg+cy MPA 10mg CEE 0.625mg+cc MPA 2.5mg CEE +cy MP 200 mg Placebo	895											→ → → →	↓ → → →	
De Souza 1996, [131]	RCT(B) 1 year	CEE 0.625mg CEE 0.625mg+ cyMPA 5 mg CEE 0.625mg+ccMPA 2.5 mg CEE 0.625mg+ccMPA 5 mg	41				→ → → →	↓ → ↓ →				↑ ↑ ↑ ↑				
Estaradiol clotting study 1996, [187]	RCT (B) 1 year	cy td E2 (50µg/24h) + cyMPA 10 mg cc td E2 (50µg/24h) + cyMPA 10 mg Placebo	188				→ ↓	→ ↓	→ →				→ ↓	→ →	→ ↓	→ →
Scarabin 1997, [114]	RCT (O) 6 months	E2 2mg + cyMP 200mg td E2 25mg/d + cyMP 200mg	44	→ →	↑ →		↓ →		→ →			→ →	→ →		→ →	↓ →
Conard 1997, [119]	RCT (B) 6 months	E2 2mg Placebo	36	→			→	→	→						↓	→
Koh 1997, [120]	RCT Cross over 1 month	CEE 0.625mg CEE 0.625mg + 2.5 mg MPA td E2 0.1mg/d td E20.1mg/d + 2.5mg MPA	50	→ →												↓ ↓ → →
Høibraaten 2000, [188]	RCT(O) 2 years	td E2 (50:g/24h) + lcy 5mg MPA Control	118	→	→	→	↓	↓	↓	→		→	→		→	↓
Teede 2000, [115]	RCT (B) 6 weeks	ccE2mg+ 1 mg NETA Placebo	42	↑	↑											↓
Van Baal 2000, [116]	RCT (B) 12 weeks	cyE2 2mg+trimegestone O.5mg cyE2 2mg+dydrogestone 10 mg cc E2 2mg Placebo	65	→ → →	↑ ↑ ↑	→ → →	→ → ↓	↓ ↓ ↓	↓ ↓ →			→ → →	↑ ↑ ↑			↓ ↓ ↓
Vehkavaara 2001, [117]	RCT (B) 12 weeks	ccE2 2mg td E2 (50:g/24h) Placebo	27	↑ →	↑ →							↑ →	↑ →	→ →	→ →	↓ →

Author	Design	Treatment	N	DD	F 1+2	TAT	AT	PS	PC	TFPI	APC-R	FVIIa	FVII	FVIII	Fg	PAI-1
Høibraaten 2001, [118]	RCT (B) 2 years	ccE2 2mg+NETA 1mg Placebo	140	↑	↑	↑	↓	↓	↓	↓		↓	→	→	→	
Høibraaten 2001, [69]	RCT (B) 2 years	ccE2 2mg+NETA 1mg Placebo	140								↑ ETP					
Peverill 2001, [122]	RCT (B) 6 weeks	ccE2mg+ 1 mg NETA Placebo	42			↑	→			↓		↑	→			
Luyver 2001, [138]	RCT (B) 3 months	CEE 0.625mg Placebo	26							↓					→	→
Demirol 2001, [139]	RCT (B) 6 months	CEE 0.625mg+MPA 5mg Placebo	110				↑				↓	↓		↓	↓	
Gottsater 2001, [130]	RCT (B) 3 months	ccE2 valerate 2mg Placebo	27 24		→		↓	↓	→		→		↑	→	↓	↓
Lobo 2001,[140]	RCT (B) 6 weeks	0.625 CEE 0.625 CEE/2.5 MPA 0.45 CEE 0.45 CEE/2.5 MPA 0.45 CEE/1.5 MPA 0.3 CEE 0.3 CEE/1.5 MPA placebo.	749				→ ↓ ↓ → → ↓ ↓	↓ ↓ ↓ ↓ ↓ ↓ ↓	↓ ↓ → ↓ → → →						↓ ↓ ↓ ↓ ↓ ↓ ↓	↓ ↓ ↓ ↓ ↓ ↓ ↓
Bladbjerg 2002, [145]	Observ. and RCT (CDCS)	cyE2 valerate 2mg+ cyCPA 1mg cyE2 valerate 2mg+ cyMPA 5mg ccE2 2mg+NETA 1mg ccE2 2mg+IUD levonorgestrel control	30 45 35 35 30							↓ ↓ ↓ ↓			→ → → →			
Salobir 2002, [121]	RCT (B) 6 months	ccE2 2mg+NETA 1mg Placebo	61	↑	→	→	↓		↓							↓
Post 2002, [134]	RCT (B) 12 weeks	cyE2 2mg+trimegestone O.5mg cyE2 2mg+dydrogestone 10 mg cc E2 2mg Placebo	65								↑ ETP ↑ ETP ↑ ETP			→ → →		
Post 2003, [152]	RCT (B) 13 cycles	E2 1mg E2 1mg+gestodene 25µg td E2 (50µg/24h) Placebo	37 33 33 49					↓ ↓ ↓	→ ↓ →		↑ ETP ↑ ETP → ETP					
Post 2003, [154]	RCT (B) 13 cycles	E2 1mg E2 1mg+gestodene 25µg td E2 (50µg/24h) Placebo	37 33 33 49	→ ↑ →	→ → →	↓ → →							↓ ↓ →		↓ → →	↓ ↓ ↓
Zegura 2003, [189]	RCT (B)	E2 2 mg td E2 (50µg/24h)	20 21	→ →	→ →	→ →									↓ ↓	↓ →
Oger 2003, [151]	RCT (B) 6 months	E2 1mg+100mg td E2 (50µg/24h) Placebo	63 68 65		↑ →			↓ →		↓ →	↑ ETP → ETP					

Author	Design	Treatment	N	DD	F 1+2	TAT	AT	PS	PC	TFPI	APC-R	FVIIa	FVII	FVIII	Fg	PAI-1
Bladbbjerg 2003, [71]	RCT (O) 5 years(DOPS)	ccE2 2mg+NETA 1mg or E2 2mg Control	187 249							↓						
Borgfeldt 2004, [190]	RCT (B) 6 months	ccE2 1mg+NETA 0.5mg ccE2 1mg+NETA 0.25mg Placebo	120				↓ ↓						↓ ↓		↓ ↓	↓ ↓
Koh 2004, [191]	RCT (B-crossover) 2 months	CEE 0.625mg + 100 mg MP CEE 0.3mg + 100 mg MP Placebo	57		↑ →		↓ ↓								→ →	↓ ↓
Pripp 2004, [192]	RCT (B) 12 months	CEE 0.625mg + 2.5 mg MPA Placebo	28	→		→	↓						↑		→	↓
Stevenson 2004, [193]	RCT (B) 6 months	td E2 (50µg/24h)+NETA 0.125mg Placebo	55	↑	→								↓		→	↑
Brynhildsen 2005, [194]	RCT (B) 48 weeks	td E2 (25µg/24h)+NETA 0.125mg Placebo	266				↓						↓		→	→
Zegura 2006, [173]	RCT (B) 6 months	ccE2 2 mg ccE2 2 mg+NETA 0.5mg td E2 (50µg/24h) Placebo	112	→ → →	→ → →	→ → →	→ → →		→ ↓ →						↓ → ↓	↓ → →
Basurto 2006, [195]	RCT (O) 3 months	Intranasal E2 (300µg) CEE 0.312mg	32				→ →	→ →	→ →			→ ↓			→ →	↕
Taner 2006, [196]	RCT (O) 6 months	CEE 0.625mg CEE 0.625mg + 2.5 mg MPA td E2 (50µg/24h) ccE2 2mg +NETA 1mg	88												↓ → → →	↓ ↓ → →
Eilertsen 2006 and 2007 (paperI,II and III)	RCT (O) 2,6,12 weeks	ccE2 2mg+NETA 1mg ccE2 1mg+NETA 0.5mg Tibolone 2.5 mg Raloxifene 60 mg	202	→ ↑ ↕	→ ↑ ↑ →	↓ ↓ → ↓	↓ ↓ ↓ ↓	→ ↓ ↓ →	↓ ↓ ↓ ↓	↓ ↓ → ↓	↑ ETP ↑ ETP → ETP ↑ ET	→ ↓ ↓ →	↓ ↓ ↓ →	→ → ↓ ↑	↓ → → ↓	→ → ↑ ↑
Brosnan 2007, [197]	RCT (O) 6 months	td E2 (25µg/24h)+NETA 0.125mg ccE2 1mg+NETA 0.5mg	344		→ →		↓ ↓				→ →		↓ ↓		→ ↓	→ ↓
Norris 2008, [198]	RCT (B) 6 months	cyE2 2mg +trimegestone 0.5mg cyE2 2mg+dydrogestone 10 mg	186	↑ ↑	→ →	→ →	↓ ↓	↓	↓ →		↓ ↓					
Hemelaar 2008, [199]	RCT (B) 12 months	Intranasal E2/NET 175/275µg ccE2 1mg+NETA 0.5mg	90	↑ ↑	→ ↑	→ →						→ ↓	↓ ↓		→ ↓	→ ↓
Fait 2008, [200]	RCT, cross 12 weeks	E2 2mg td E2 (50µg/24h)	45	→ →			↓ ↓			↓ →					↓ →	↓ →

Abbrevations:RCT: Randomized clinical trial, B:blinded, O: Open, Cy cyclic, cc: continous combined, td:transdermal, E2: estradiol, CEE: Conjugated equine estrogens (CEE), NETA: northisterone acetate, MP: micronized progesterone, MPA: medroxyprogesterone acetate, APC-SR: activated Protein C sensitivity ratio, DD:D-dimer, Fg:Fibrinogen, ETP: endogenous thrombin potential ↑:Increase, ↓ : Decrease, →: No change. Studies published prior to the RET study in blue fill.

Table 4 Impact of HT on markers of activated coagulation, coagulation factors and inhibitors in randomized studies

Author	Design	Treatment	N	DD	F 1+2	TAT	AT	PS	PC	TFPI	APC-R	FVIIa	FVIIag	FVIII	Fg	PAI-1
Van Wersch 1994, [126]	RCT (O) 6 and 12 months	Tibolone 2.5 mg cyEstradiol valerate (E_2V) 2mg +cy-cyproterone actate 1mg	60	→ →	→ →	→ →							→ →		→ →	↓ →
Winkler 2000, [123]	RCT (B) 12 , 24 weeks	Tibolone 2.5 mg ccE2 2mg+ccE3 1mg+NETA 1mg	29 31	→ ↑	→ ↑	→ →	→ ↓	↓ ↓	→ ↓		↑ →	↓ ↓			→ ↓	
Norris 2002, [124]	RCT (O) 3, 6, 12months	Tibolone 2.5 mg ccE2 2mg+NETA 1mg	80	→ →		→ →	↓ ↓						↓ →		→ →	↓ ↓
Koh 2003, [125]	RCT (B-crossover) 2 months	Tibolone 2.5 mg CEE 0.625mg + 100 mg MP Placebo	82		↑ ↑		↓ →								→ →	↓ ↓
Koh 2005, [201]	RCT (B crossover) 2 months	Tibolone 2.5 mg CEE 0.3mg + 100 mg MP Placebo	41		↑ →		→ ↓								→ →	↓ →
Osmanağaoğlu 2005, [147]	RCT(O) 6 months Obese women	Tibolone 2.5 mg CEE 0.625mg + 2.5 mg MPA ccE2 2mg+NETA 1 mg	352				→ → →	→ → →	→ → →				→ → →	→ → →	↓ ↓ ↓	
Eilertsen 2006 and 2007 (paperI,II and III)	RCT (O) 12 weeks	ccE2 2mg+NETA 1mg ccE2 1mg+NETA 0.5mg Tibolone 2.5 mg Raloxifene 60 mg	202	→ ↑ ↕ ↓	→ ↑ ↑ →	↓ ↓ → ↓	↓ ↓ ↓ ↓	→ ↓ ↓ →	↓ ↓ ↓ ↓	↓ ↓ → ↓	↑ ETP ↑ ETP → ETP ↑ ETP	→ ↓ ↓ →	↓ ↓ ↓ →	→ → ↓ ↑	↓ → → ↓	→ → ↑ ↑
Skouby 2007, [202]	RCT 12 months	Tibolone 1.25 mg and 2.5 mg CEE 0.625mg + 5 mg MPA	38				→ ↓	→ ↓	↓ →	→ ↓	↑ ↑					↓ ↓
Demirol 2007, [203]	RCT(B) 24 weeks	Tibolone 2.5 mg CEE 0.625mg Placebo	90				→ ↑				→ →	↓ ↓		→ →	→ ↓	
Keramaris 2007, [204]	RCT (O) 12 months	Tibolone 2.5 mg CEE 0.625mg CEE 0.625mg + 5 mg MPA ccE2 1mg+NETA 0.5 mg control	24 24 34 66 68				→ ↓ ↓ ↓	→ → ↓ →	→ ↓ ↓ →							

Abbrevations:RCT: Randomized clinical trial, B:blinded, O: Open, Cy cyclic, cc: continous combined, td:transdermal, E2: estradiol, CEE: Conjugated equine estrogens (CEE), NETA: northisterone acetate, MP: micronized progesterone, MPA: medroxyprogesterone acetate, APC-SR: activated Protein C sensitivity ratio, DD:D-dimer, Fg:Fibrinogen, ETP: endogenous thrombin potential ↑:Increase, ↓ : Decrease, →: No change. Studies published prior to the RET study in blue color.

Table 5 Impact of Tibolone on markers of activated coagulation, coagulation factors and inhibitors in randomized studies

Author	Design	Treatment	N	DD	F 1+2	TAT	AT	PS	PC	TFPI	APC-R	FVIIa	FVIIag	FVIII	Fg	PAI-1
Walsh 1998, [142]	RCT (B) 6 months	Raloxifene 60 mg Raloxifene 120 mg CEE 0.625mg +cc MPA 2.5mg Placebo	84 92 83 90		→ → →										↓ ↓ →	→ → ↓
De Valk-de Roo 1999, [127]	RCT (B) 24 months	Raloxifene 60 mg Raloxifene 120 mg CEE 0.625mg Placebo	15 15 15 15	→ → →	→ → ↑	→ → →							→ → →		↓ ↓ ↓	→ → ↓
Nickelsen 2001, [141]	RCT (B) (Euralox1) 6 months	Raloxifene 60 mg ccE2 2mg+NETA 1mg	495 513												↓ ↑	
Duschek 2004, [153]	RCT (B) 24 months	Raloxifene 60 mg Raloxifene 150 mg CEE 0.625mg +cc MPA 2.5mg Placebo	23 20 17 23								→ ETP → ETP ↑ ETP					
Cosman 2005, [128]	RCT (B)	Raloxifene 60 mg Tamoxifene 20 mg CEE 0.625mg Placebo	24 24 23 23	→ → ↑			→ ↓ →	→ → ↓	→ ↓ →		→ → →		→ → ↑	→ ↑ ↑		→ → →
Dias 2005, [205]	RCT (B) 4 months	Raloxifene 60 mg CEE 0.625mg Placebo	90				↓ ↓		↓ ↓						↓ ↓	→ →
Eilertsen 2006 and 2007 (paperI,II and III)	RCT (O) 2,6,12 weeks	ccE2 2mg+NETA 1mg ccE2 1mg+NETA 0.5mg Tibolone 2.5 mg Raloxifene 60 mg	202	→ ↑ ↓	→ ↑ →	↓ ↓ ↓	↓ ↓ ↓ ↓	→ ↓ ↓ →	↓ ↓ ↓ ↓	↓ ↓ → ↓	↑ ETP ↑ ETP → ETP ↑ ETP	→ ↓ ↓ →	↓ ↓ ↓ →	→ → ↓ ↑	↓ → → ↓	→ → ↑ ↑
Sgarabotto 2007, [158]	BRCT 12 months	Raloxifene 60 mg ccE2 2mg+NETA 1mg	30 24	→ →	↑ ↑	→ →	↓ ↓	→ →	→ →		↓ ↓		→ →	↑ ↑	↑ ↑	→ →

Abbrevations:RCT: Randomized clinical trial, B:blinded, O: Open, Cy cyclic, cc: continous combined, td:transdermal, E2: estradiol, CEE: Conjugated equine estrogens (CEE), NETA: northisterone acetate, MP: micronized progesterone, MPA: medroxyprogesterone acetate, APC-SR: activated Protein C sensitivity ratio, DD:D-dimer, Fg:Fibrinogen, ETP: endogenous thrombin potential ↑:Increase, ↓ : Decrease, →: No change. Studies published prior to the RET study in blue color.

Table 6 Impact of Raloxifene on markers of activated coagulation, coagulation factors and inhibitors in randomized studies

2. Aims and hypotheses

Several studies have demonstrated that oral HT containing estrogen in conventional doses was associated with activation of coagulation and an increased risk of VT. We wanted to investigate whether lower dose of HT and other HT regimens had differential impact on relevant markers of the coagulation and fibrinolytic system, and on inflammation. The overall aim of this thesis was to compare the effects of different HT regimens on markers of activated coagulation, and to identify potential mechanisms for activated coagulation by analyzing markers of vascular disease. We were particularly interested in evaluating a possible relationship between dose of estrogen-progestin and impact on the various markers. Change in levels of D-dimer was the primary efficacy parameter in the RET- study.

The aims of the thesis addressed in the different papers:

Compare the impact of various HT regimens on markers of activated coagulation.

- To evaluate a possible relationship between dose of estrogen-progestin and degree of activation of coagulation (paper I)
- To compare the differential effects of HT, tibolone and raloxifene on coagulation activation (paper I)

To elucidate possible mechanism(s) for differences in coagulation activation

- To study the effect on coagulation factors, coagulation inhibitors, fibrinolytic factors, and the association with markers of activated coagulation (paper II)
- To study whether the different regimens induce an APC resistant phenotype (paper III)
- To examine the effects of the different regimens on CRP and other markers of inflammation, and their association with markers of activated coagulation (paper IV)

To compare different routes of administration in women with high risk of vascular disease

- To study the effects of oral and transdermal HT on CRP, and other markers of inflammation in women with high risk of thrombosis (paper V)

3. Methods

This thesis is based on three single-centre RCTs; The Raloxifene, Estrogen and Tibolone (RET) study (papers I, II, III, and IV); The Estrogen in Venous Thromboembolism Trial (EVTET) and the Estrogen Women Atherosclerosis (EWA) study (paper V). Various biochemical markers and risk indicators related to venous and arterial disease were assayed. All the studies were conducted at the Department of Hematology at the Ullevål University Hospital Trust, Oslo.

3.1 Study design, subjects and intervention

3.1.1. Raloxifene, estrogen and tibolone (RET) study

This study was conducted from December 2002 to January 2005, and was designed as an open-label, randomized, comparative clinical study with four parallel groups. Change in levels of D-dimer was the primary efficacy parameter. In this study, 202 healthy postmenopausal women between the age of 45 and 65 years were included. Inclusion and exclusion criteria are described in detail in paper I. Women with a history of VT or symptomatic atherosclerotic disease or known thrombophilia were not included. The women were randomly allocated to four treatments; conventional-dose HT (2 mg E2 and 1 mg NETA), *n=50*; low-dose HT (1 mg E2 and 0.5 mg NETA), *n=50*; tibolone (2.5 mg tibolone), *n=51*; or raloxifene group (60 mg raloxifene hydrochloride), *n=51*.

3.1.2. The Estrogen in Venous Thromboembolism Trial (EVTET)

Between February 1996 and September 1998, 140 postmenopausal women were included in this double-blind placebo-controlled RCT. The women were under the age of 70 years, and had previously at least one verified VT, i.e., DVT or PE. The study design and main results have been reported in detail earlier [21]. The major outcome parameter, recurrent VT, occurred in eight women in the treatment group and in one woman in the placebo group. The study was terminated prematurely due to circumstantial evidence emerging during the trial. The women received treatment for 24 months with once daily either placebo tablets (*n=69*) or HT tablets containing 2 mg E2 and 1 mg NETA (*n=71*).

3.1.3. The Estrogen Women Atherosclerosis (EWA) study

In this open controlled randomized study, 118 postmenopausal women were included between May 1995 and January 1997. The women were younger than 71 years and had angiographically verified CAD. The study design and primary outcome have been reported in detail earlier [206]. The women were randomized to receive either transdermal HT (*n=60*) given as unopposed transdermal E2 (50 μg/24h), with sequential administration of 5 mg tablets of MPA once daily for 14 days every 3 months, or to a control group receiving no therapy (*n=58*).

3.2 Data collection

Details on data collection procedures in the three studies are described elsewhere; RET-study (paper I), EVTET [21] and EWA [206]. Venous blood samples were collected before treatment (at baseline) and at follow-up visits. In the RET-study visits were scheduled after 2, 6 and 12 weeks. In the EVTET-study the follow-up visits were after 3, 12 and 24 months, and in the EWA-study after 3 and 12 months. Women who discontinued before schedule due to adverse events, or withdrew their consent to participate, were followed until recovery of adverse event or until the time of consent withdrawal.

3.3 Laboratory Assays/Measurements

Citrated plasma was used to measure markers of activated coagulation (paper I), levels of coagulation factors, coagulation inhibitors and markers of fibrinolysis (paper II). Markers of inflammation (paper IV and paper V) were analyzed in serum. All markers were analyzed with enzyme-linked immunosorbent assays (ELISA). Commercial kits were used and run as described by the manufacturers. Further details are given in the separate papers of this thesis.

The function of the APC system was analyzed by testing the effects of recombinant APC (rAPC) or thrombomodulin (rTM) on the Endogenous Thrombin Potential (ETP) as determined with the Calibrated Automated Thrombogram (CAT) assay. CAT was performed as described by Hemker et al [45]. To increase the reproducibility data were expressed as normalized APC sensitivity ratios (nAPCsr) by dividing the APCsr of test plasma with the same ratio obtained with PNP, either generated with rAPC or with rTM:

$$nAPCsr = \frac{(ETP_{+APC\ or\ TM} / ETP_{-\ APC\ or\ TM})\ test\ plasma}{(ETP_{+APC\ or\ TM} / ETP_{-\ APC\ or\ TM})PNP}$$

Thus, nAPCsr >1.0 reflects an APC resistance higher than in pooled normal plasma (PNP), which is consistent with an APC resistant phenotype. Further details are given in paper III.

3.4. Statistics

3.4.1. Demographic variables

The continuous demographic variables were approximately normally distributed and were given as mean ± standard deviation (SD), whereas categorical variables were presented as percentages. When comparing a continuous variable for more than two groups, the Kruskal-Wallis test was used. Comparison of dichotomous variables between the groups was performed with the Chi-Square test.

3.4.2 Descriptive statistics (The efficacy variables)

The efficacy variables were given as mean and 95% confidence interval (CI) when approximately normally distributed, or as median and interquartile range (25^{th} and 75^{th} percentiles) if not. Treatment effects were calculated as the change, i.e., the difference from baseline to 2, 6 and 12 weeks, respectively, in the RET-study, and after 3 and 12 months in the EVTET and EWA-studies. Depending on the degree of skewness in the distribution of the variable, the treatment effects were presented as mean or median percent change with 95% CI.

3.4.3 Significance testing

Baseline values and values after treatment were compared by using a two-sided Wilcoxon Signed Rank test. Differences in mean or median percent change between two groups were analyzed using a two-sided t-test or two-sided Mann-Whitney test when appropriate. Primary comparisons in the RET-study were performed between the effects observed in the low-dose HT group and in each of the other treatment groups. When comparing the effects

for the whole treatment period, repeated measurements ANOVA (RMANOVA) were performed after logarithmic transformation (paper II and IV). In the RET-study findings with a p-value of $\leq$0.05 were considered significant. Due to the multiple comparisons performed in the EVTET and EWA-study, a 1% significance level was applied.

3.4.4. Correlations and regression models

Correlation between two variables was expressed as the Spearman correlation coefficient. In order to identify factors associated to change in D-dimer and CRP, linear regression analysis was used. When performing regression analysis, the basic assumption underlying such analysis, e.g., normality and linearity, were checked and found to be adequately met. Further details on the regression models are described in papers II and IV.

3.4.5. Power calculation in the RET -study

D-dimer was the primary efficacy parameter in this study. Based on results obtained in the EVTET study [118], we expected a difference in change in D-dimer between the conventional- and low-dose HT groups of 60 ng/mL (SD 90 ng/mL). Approximately 40 individuals were needed in each treatment group in order to have 80% test power when comparing these two treatments. To correct for an expected 20% drop-out-rate we decided to include 50 women in each treatment group.

3.4.6. Statistical packages

Statistical analyses were performed using the Statistical Package for the Social Sciences version 11.0. (SPSS Inc., Chicago, Illinois, USA), except for the 95% CIs, which were calculated using Number Cruncher Statistical Systems (NCSS, Kaysville, Utah, USA).

3.5 Ethical aspects

The protocols were approved by the Regional Committee for Research Ethics in Health Region East, Norway, and by the Norwegian Medicines Agency. Written informed consent was obtained from each participant before inclusion. All three studies were carried out in accordance with the Helsinki declaration and Good Clinical Practice Guidelines.

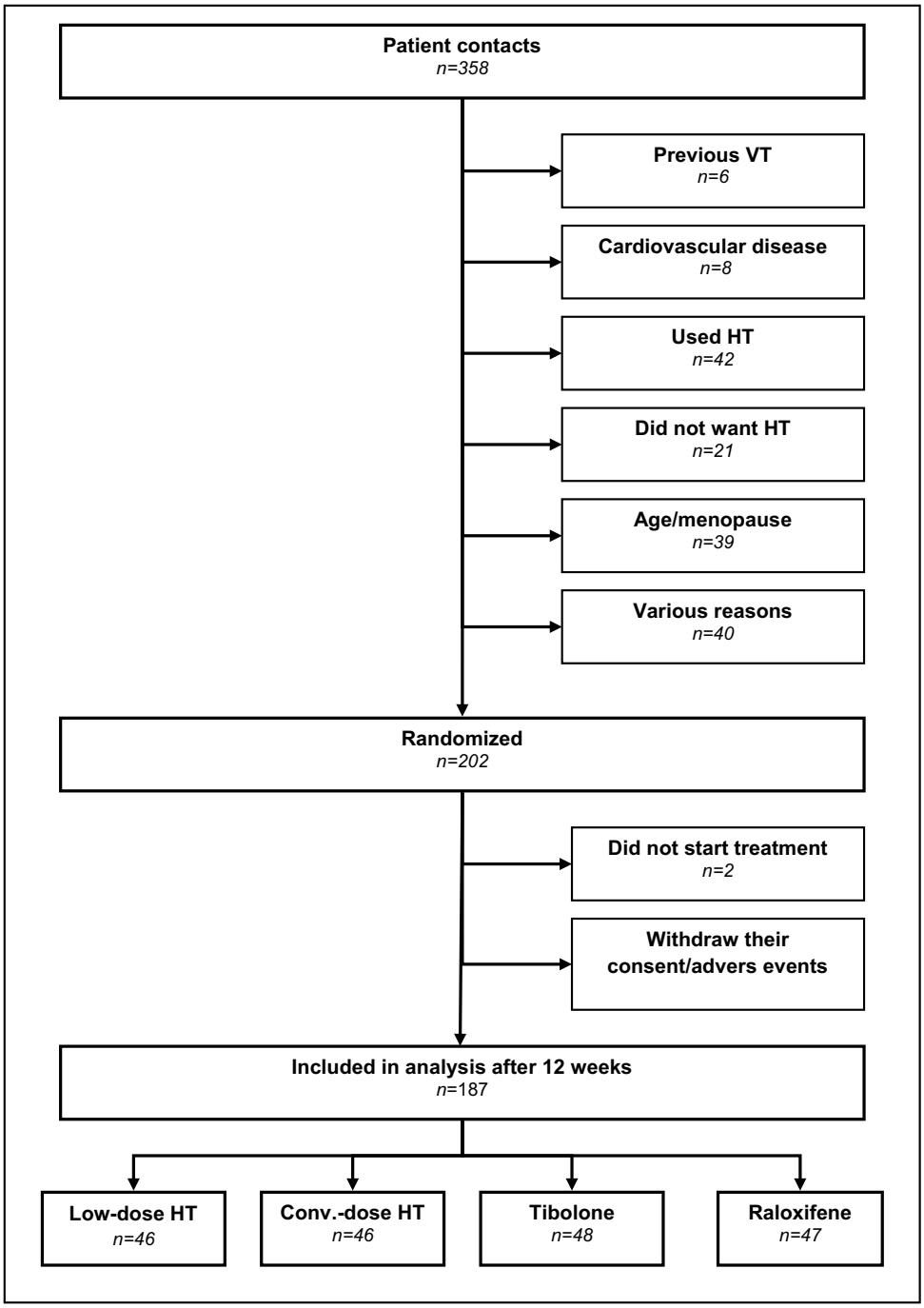

Figure 5 Flow chart in the RET-study, showing the number of women screened, number and reasons for not being included in the study

4. Main results

4.1. Demographic characteristics in the RET-study

Baseline data

Demographic characteristics (age, body mass index [BMI], smoking habits, time since amenorrhea, and blood pressure) were similar at baseline for the four treatment groups (table 4). Two women, both in the raloxifene group, did not start treatment; one due to baseline mammography findings and one due to anxiety. During the study, 13 women stopped prematurely or withdrew their consent to participate. These dropouts and exclusions were evenly distributed between the groups (low-dose HT group; *n=4,* conventional-dose HT group; *n*=4, tibolone-group; *n=3* and raloxifene group; *n=2*), and were due to known adverse effects of these regimens (table 5).

	Low-dose HT ***n=50***	**Conv.-dose HT** ***n=50***	**Tibolone** ***n=51***	**Raloxifene** ***n=51***	**P-value**
Age (years**)**	54.7 ± 5.1	56.1 ± 3.6	55.1 ± 4.7	56.5 ± 4.8	0.16
BMI (kgm^{-2})	25.4 ± 4.0	24.9 ± 3.2	25.3 ± 3.6	25.2 ± 3.2	0.92
SBP (mm Hg)	128 ± 17	124 ± 14	126 ± 14	127 ± 16	0.64
DBP (mm Hg)	83 ± 9	80 ± 8	80 ± 8	81 ± 10	0.38
Heart rate ($beats\ min^{-1}$)	69 ± 8	69 ± 8	70 ± 10	70 ± 12	0.97
Smokers (percent**)**	11 (22 %)	16 (32 %)	16 (31 %)	15 (30 %)	0.67
Natural menopause (percent)	41 (82 %)	36 (72 %)	40 (78%)	40 (80%)	0.65
Years from menopause* (years)	5.9 ± 5.5	6.3 ± 4.4	5.9 ± 4.8	7.9 ± 5.2	0.65

Values were given as mean ± SD or number and percentage of the women. The comparisons between baseline values were performed by using one-way ANOVA for continuous variables and Pearson Chi-Square for the categorical variable. BMI - body mass index; SBP - systolic blood pressure; DBP - diastolic blood pressure.
*Years since menopause for women who had a defined onset of natural menopause, n=157 (78%).

Table 7 Demographic characteristics.

	Low-dose HT *n=4*	Conv.-dose HT *n=4*	Tibolone *n=3*	Raloxifene *n=2*
Myocardial infarction	-	1	-	-
Genital bleeding	1	3	-	-
Genital discharge	1	-	-	-
Weight gain	-	-	1	-
Edema	1	-	-	-
Dizziness	-	-	1	1
Palpitations	-	-	-	1
Lost to follow up	1	-	-	-
Elevation of bilirubin	-	-	1	-

Table 8 Reasons for dropout in the RET-study

4.1 Paper I

Conventional-Dose Hormone Therapy (HT) and Tibolone, but not Low-Dose HT and Raloxifene, Increase Markers of Activated Coagulation

In this paper the impact of the four differential HT regimens on the primary efficacy markers of the study, i.e., markers of activated coagulation, was evaluated**.** D-dimer, F_{1+2} and TAT-complex were measured in 202 postmenopausal women after 2, 6 and 12 weeks of HT. D-dimer increased markedly in the conventional-dose HT group, but remained unchanged in the low-dose HT group. This difference was statistically significant (RMANOVA $p<0.0001$). Tibolone was associated with a medium increase, whereas raloxifene was associated with a decrease in D-dimer levels. The changes in the raloxifene group differed significantly from the low-dose HT group (RMANOVA, $p<0.0001$), whereas the changes in the tibolone group did not. Changes in F_{1+2} showed a similar pattern for all four groups. However, the differences in F_{1+2} between the groups were only significant between the low-dose and the conventional-dose group (RMANOVA $p=0.003$). No significant differences in changes of thrombin-antithrombin complex were observed.

4.2 Paper II

Differential effects of conventional and low dose oral hormone therapy (HT), tibolone, and raloxifene on coagulation and fibrinolysis

To elucidate the possible mechanisms for the activated coagulation, we investigated the effect of four HT regimens on selected clotting factors, coagulations inhibitors and

fibrinolytic factors. A multivariate regression model was used to determine their contribution to activation of coagulation. The conventional- and low-dose HT groups generally showed similar effects, i.e., reductions in both clotting factors and inhibitors, but the effects were more pronounced in the conventional-dose HT group. Compared with the low-dose HT group, those treated with tibolone showed more pronounced decreases in factor VII, less reduction of AT and PC, and even increased levels of PS and TFPI. As opposed to the low-dose HT group, in the raloxifene group the reductions in inhibitors were smaller. Moreover, in those allocated to raloxifene, reduced levels of fibrinogen were seen, whereas other coagulation factors and PAI-1 were increased. In the conventional-dose HT group a decrease in FVII antigen was significantly related to a decrease in D-dimer (6 weeks: $p=0.029$), whereas in the tibolone-group a decrease in TFPI was significantly related to an increase in D-dimer (12 weeks: $p=0.025$). In the raloxifene group reduction in fibrinogen was a significant predictor for reduction in D-dimer (6 weeks: $p<0.0001$, 12 weeks: $p=0.001$).

4.3 Paper III

Differential impact of conventional and low dose oral hormone therapy (HT), tibolone and raloxifene on functionality of the activated protein C system

To study the possible mechanisms for the activation of coagulation, we analyzed the effect of the HT regimens on the sensitivity to APC in an assay with high sensitivity for hormonal changes. NAPCsr were determined in plasma collected at baseline and after 12 weeks, using a thrombin generation-based APC resistance test probed with either rAPC or by rTM. NAPCsr increased in both the conventional- and low-dose HT groups, consistent with reduced sensitivity to APC. The increase was slightly more pronounced in the conventional-dose group, but the difference between the two HT groups was not statistically significant (mean difference -0.13). We observed an increase in nAPCsr probed with rTM, but there were no differences between the conventional- and low-dose HT groups (mean difference -0.004). The sensitivity to APC was only marginally altered in those allocated to tibolone (mean difference 0.39 and 0.19 when probed with rAPC and rTM, respectively). Consequently, tibolone showed a different phenotype as compared with the low-dose HT group. A small increase in nAPCsr with both rAPC and rTM was seen in the raloxifene-group, but the increase was less than in the low-dose HT group (mean difference 0.23 and 0.13 when probed with rAPC and rTM, respectively). There was a negative correlation ($r=-0.22$,

p=0.002) between baseline values of D-dimer and nAPCsr generated with TM. In the low-dose HT and tibolone groups the change in nAPCsr (with rAPC), was negatively correlated to the change in F_{1+2} (r=-0.37, p=0.01, and r= -0.35, p=0.02, respectively), whereas there was no correlation between these parameters in the conventional-dose HT and raloxifene groups. Neither was there a correlation between change in nAPCsr and change in D-dimer.

4.4 Paper IV

Differential impact of conventional dose and low dose hormone therapy (HT), tibolone and raloxifene on C-reactive protein (CRP) and other inflammatory markers

In this paper, the impact of the four different HT regimens on CRP and other inflammatory markers was compared. Furthermore, the possible underlying mechanisms for changes in CRP and D-dimer were investigated. CRP increased in the conventional and low-dose HT group. These changes were more pronounced in the conventional-dose group (RMANOVA p=0.02). In addition, tibolone was associated with an increase in CRP, in contrast to raloxifene, which reduced CRP. None of these changes was significantly different from the low-dose HT group. Reduction in levels of Lp (a), ICAM, P-selectin, E-selectin and MCP-1 were observed in all the four treatment groups. The changes were most pronounced for the conventional dose HT group, least pronounced for the raloxifene, whereas the changes in those allocated to tibolone and low-dose HT were intermediary. IL-6 decreased in all groups, but none of the changes was significantly different from the low-dose group. As opposed to the other groups, increased levels of TNF-α and VWF ag were seen in the raloxifene group, and these changes were different from the low-dose group. The average increase in CRP was not accompanied by an increase in average IL-6 or TNF-α or other markers, but women with large reductions in IL-6 had reduced increase in CRP.

4.5 Paper V

The effects of oral and transdermal hormone replacement therapy on C-reactive protein levels and other inflammatory markers in women with high risk of thrombosis

In paper V, we compared the effects of oral and transdermal HT on CRP levels in women with high risk of thrombosis. In EVTET, additional inflammatory markers were assayed i.e., IL-6, TNFα, TGFβ, P-selectin, and VCAM-1. In the EVTET study, which included 140 women with a history of VT, oral HT was associated with strong activation of coagulation markers

and increased risk of recurrent VT [21]. No such associations were observed in the EWA-study, where 118 women with established coronary artery disease were given transdermal HT [188]. Oral HT was associated with a significant increase in CRP after 3 months as compared with placebo (p=0.001). These changes sustained after 12 months. Among those allocated HT the median increase in CRP was higher in women who subsequently developed recurrent VT (median 328%, n=5, versus 54%, n=60). Levels of TNFα and VCAM-1 decreased significantly in the HT group as compared to the placebo group (p=0.004 and p<0.001, respectively). There were no significant changes in levels of IL-6, TGFβ or P-selectin. On transdermal HT, no significant changes in CRP were observed after 3 and 12 months of treatment.

5. General discussion

5.1 Methodological considerations

5.1.1 Study design

The RET study was an open-label, randomized clinical study, designed to compare the impact of four different hormone regimen on hemostasis. The main goal of a RCT is to compare groups of patients who only differ with respect to their treatment. The methods applied in these studies aim to eliminate bias. The random allocation ensured that treatment of the patients was carried out in an unbiased way. However, the open design in the RET-study did not allow us to rule out the bias connected to adherence and follow-up. However, both HT and raloxifene are frequently accompanied by minor side effects that render valid blinding difficult. Hence, the open design regarding treatment may on the contrary have contributed to the low dropout rate observed in our study (figure 5 and table 8). I would like to emphasize that the efficacy parameters in this study were changes in levels of biochemical markers, and not clinical end-points, such as clinical symptoms or frequency of disease. The overall aim of this study was to evaluate the impact of different HT on levels of activated coagulation and to identify potential mechanisms for activation. It could be argued that a more optimal design would have been a placebo-controlled study. However, this would entail significant increase in cost of conducting the study, which was not feasible with the limited funding of the study. Moreover, since all the biochemical markers were analyzed in a blinded fashion it is rather unlikely that the open design have influenced the test results.

Another potential weakness is the lack of an untreated control group in the RET-study. A control group would have been beneficial in comparing our results in the low-dose group, to interpret these findings, and would probably have allowed us to conclude stronger on our findings in general. Nevertheless, the RET-study was designed to compare the four groups and the study was adequately powered (see sample-size calculation in Methods) to detect differences in change of D-dimer between the groups.

Our data is limited to measuring impact of treatment after a given time period (12 weeks in the RET-study), which does not allow us to conclude on the long term effects of these treatments. However, in the EVTET study the levels of D-dimer and other markers measured after 3 months sustained after 12 months.

5.1.2 Study population

The RET study contains a heterogeneous population of healthy postmenopausal women; both new and prior users of HT were included. Although the groups were comparable regarding important demographic characteristics at baseline (table 7), we can not exclude the possibility that heterogeneity may have influenced the effects of treatment on various biochemical markers.

Since the efficacy parameters were biochemical markers of vascular disease, we included healthy women with all degrees of climacteric symptoms in the study. The clinical indication for use was not recorded at inclusion and not measured nor evaluated during the study. However, the women included in our study probably reflect the population, who according to age and physical health were eligible for this treatment. Hence, our results are probably valid in the general population and have a potential for utilization in clinical practice.

The EVTET study revealed an increased risk of recurrent VT by use of HT in women with previous VT [21]. These results clearly indicate that these women should avoid HT, and have thereby made substantial contribution to clinical decision making. The findings in the EWA and EVTET studies are limited to women with high risk of thrombosis. Due to increased risk of thrombosis in women with previous vascular disease demonstrated in EVTET and other recent studies, it now appears unethical to perform RCTs on these women.

5.1.3 Intervention

In order to assess the relation between dose and impact on the various biochemical markers, different doses of HT were studied. To more thoroughly evaluate a potential dose-response relationship between the regimens containing E2 and NETA, we originally wanted to include a treatment group with even lower doses of estrogen and progestin, i.e., 0.5 mg E2 and 0.1 mg NETA. Unfortunately, preparations with lower doses were not available at the start of inclusion of this study.

To study the underlying mechanism(s) for the activated coagulation, we compared tibolone and raloxifene with combined HT. This was based on the knowledge that these regimens have differential chemical structure and affinity for the various steroid receptors (see introduction). We postulated that these differences might reveal differences in impact on markers related to vascular disease.

The first study reporting differential risk of VT between oral and transdermal therapy was published during the time period we included women in the RET-study [84], and has later been confirmed by another case-control study [85]. The beneficial profile of transdermal therapy is supported by several studies on biochemical markers [117;151;152;177-180;188], and by our findings on markers of inflammation in women with high risk of thrombosis (paper V).

In the RET-study the serum levels of E2, tibolone and raloxifene were not measured. The bioavailability of treatments in each group can therefore not be confirmed.

5.1.4 Measurements

The primary and secondary outcomes of the RET-study were biochemical markers of vascular disease. In the EVTET study, the increased risk of VT was related to a marked activation of coagulation, as measured by D-dimer and F_{1+2} [118]. These changes are suggested to be attributed to reduced levels of coagulation inhibitors, e.g., TFPI and acquired APC resistance [69]. The hypotheses of the RET-study were based on these results. Hence, the hemostatic factors assayed were mainly the same. The selection of biochemical markers related to endothelial function and inflammation was based on the circumstantial evidence in the field.

To measure the different markers, mainly commercially available ELISA-kits were chosen. The ELISA methods are extensively tested and show low test-retest variability. Interassay coefficients of variation (CV) were between 2.1% and 12.9% for the various assays. All analyses were performed by skilled technical staff with long experience in our research laboratory. All assays were performed examiner-blind, and the samples were run in batch by the end of the study using a balanced set-up with an equal number of samples from the four different groups and with all samples for the same individual in each run.

To evaluate the impact on functionality of the APC-system, a thrombin generation based APC-resistance test was used. This test has shown to be more sensitive to changes in hormonal changes than an APTT-based assay [207]. Even though CAT is an automated method, many of the processes involved are subjected to day-to-day variability. To increase the reproducibility, data were expressed as nAPCsr by dividing the APCsr of test plasma with the same ratio obtained with PNP (see Methods).

5.1.5.Statistical aspects

Most of the variables had skewed distributions. The efficacy parameters were expressed as change from baseline in percent (%), with both negative and positive results, and logarithmic transformation was not possible. Non-parametric statistical methods were therefore applied. When the efficacy parameters were suitable for logarithmic transformation, parametric methods were chosen. To reduce the possibility of false positive results, we performed multivariate repeated measures analysis (RMANOVA).

There are some inconsistencies in the statistical methods applied in the different papers. This is mostly due to different suggestions by reviewers during the peer-review process of publication. An example is the presentation of mean change in nAPCsr in paper III, as opposed to the other papers presenting median percent change of the efficacy variable.

5.2 Discussion of main findings

5.2.1 Impact of hormone therapy on activation of coagulation

To our knowledge the RET-study was the first study to compare the impact of conventional- and low-dose HT containing E2 on markers of activation of coagulation. However, a relationship between dose of estrogen and effect on markers of activated coagulation was suggested in a small study from the early 1990-ies, which evaluated conventional (0.625 mg) and high dose (1.25 mg) CEE treatment [112]. More recently, another report of similar impact of dose of HT on coagulation from a study of oral HT containing CEE and MP was published [191].

The findings of strong activation of coagulation in the conventional-dose HT group are in line with several other studies, in which an activation of the coagulation system has been observed on conventional-dose HT in both healthy women [112-117] and women with a history of VT [118]. Two recent studies investigating low-dose HT, containing 1 mg E2 combined with gestodene or MP, have measured some activation of coagulation as determined with D-Dimer [154] or F_{1+2} [151]. Our finding, with no significant activation of coagulation in the low-dose HT group, is supported by a recent publication by Brosnan et al [197] utilizing the same low dose HT regimen as in our study, and by a study by Koh et al, which evaluated 0.3 mg CEE and MP [201] (table 4).

5.2.2 Impact of tibolone and raloxifene on activation of coagulation

In paper I, we reported that tibolone, though to a lesser extent than conventional dose HT, also changed the overall hemostatic balance towards a more active state, with increased levels of D-dimer and $F_{1+2.}$ Similar effects of tibolone on D-dimer have been reported in two other studies [123;124], whereas another did not detect any changes [126]. Studies on F_{1+2} have shown conflicting results (table 5) [123;125;126;201].

In the RET-study, we observed reductions in levels of D-dimer and a neutral effect on the other markers of coagulation activation in those allocated to raloxifene. Studies on raloxifene have shown no significant changes in D-dimer and TAT [127;128;158], and conflicting results regarding F_{1+2} (table 6) [127;142;158].

5.2.3 Impact of hormone therapy on coagulation

Our study is in line with previous studies showing that oral HT affects all known pathways of coagulation inhibition [112-114;116;118;122;129;130;132;144-146;151;152]. Interestingly, the reductions in coagulation inhibitors, i.e., AT, PC and TFPI, were significantly less pronounced in the low-dose HT group. This dose-dependent effect on coagulation inhibitors may explain the diversity in activation of coagulation between low and conventional doses of HT. The relation between dose given and effects observed is also apparent for effects on coagulation factors and markers of fibrinolysis. Furthermore, we observed more pronounced reductions in sensitivity to APC in the conventional-dose HT group, but as opposed to many other markers the differences between the two HT groups were not statistically significant. A clearer relation to dose might have been revealed if lower doses of HT had been tested or if the sample size was larger. A post-hoc power calculation showed that the true difference in nAPCsr may be as large as 0.15 even if the observed difference between the groups in our study was non-significant (further details in paper III).

5.2.4 Impact of tibolone and raloxifene on coagulation

Our study is in line with other studies demonstrating reduced levels of AT after treatment with tibolone [123;124]. As recently demonstrated in another study [202], we observed reductions in PC, while others have shown no change [123;204]. In contrast to other studies showing reduction or no effect on PS [123;147;202;204], we observed increased free PS levels. Moreover, our study showed that tibolone increased TFPI activity, but had only minor

effects on the other TFPI assays. Tibolone only marginally altered nAPC-SR in our study, while others have demonstrated increased APC resistance ratio [123;202]. Consequently, compared with HT, tibolone shows a different phenotype with regard to APC. The differential effects on inhibitors between HT combinations and tibolone may be related to the androgenic properties of tibolone. This is supported by observations that men have higher levels of TFPI and PS than women. Moreover, androgens have been shown to increase expression of TFPI-genes in male macrophages [208].

Studies evaluating the effects of raloxifene on coagulation inhibitors have shown reduction in AT [128;148-150;158]. There are few reports on PC and PS, but our results are in line with the most well-powered of these [128]. No clinical studies evaluating the effects of raloxifene on TFPI are available. Studies evaluating the effect of raloxifene on APC resistance have shown conflicting results, i.e., either no effect or reduced sensitivity to APC as in our study [128;149;153].

5.2.5 Impact of hormone therapy on inflammation

Our findings in the conventional-dose group are consistent with the results of several other studies, which have reported increased CRP levels on HT in equivalent doses, i.e., 2 mg E2 or 0.625 mg CEE [135;138;155;161-163]. Other studies have reported no significant effects on CRP [165;174-176;209]. The discrepancy between these studies may be explained by the different type or dose of estrogen or progestin. Some of these studies suggest that regimens with lower doses of estrogen to a lesser extent increase the CRP levels [174-176], which are in line with our findings in the low-dose HT group. Moreover, our findings that transdermal HT induced no changes in CRP (paper V) are in agreement with other studies [179].

In concordance with other studies, we observed reductions in ICAM, P-selectin and E-selectin in both HT-groups [160;165;167-171] suggesting an anti-inflammatory effect of HT on the endothelium. HT decreased levels of MCP-1 in our study, which is in line with other studies [166-168].

5.2.6 Impact of tibolone and raloxifene on inflammation

Only a limited number of other studies have evaluated the effect of tibolone on CRP [125;181-184]. Our results are in line with the majority of these [181;184], and the studies showing no effects on CRP were small and thus probably underpowered [125;182]. Some

studies have indicated that CRP levels are unaffected by raloxifene treatment [127;169;185;186], whilst we observed small, but significant reductions.

5.2.7 Proposed mechanism of activation of coagulation

Despite extensive evaluation of the effects of HT and estrogen analogues on various markers of coagulation, the mechanism(s) for the increased risk of VT remain unresolved. Unraveling these mechanisms is challenging, due to the use of many different HT formulations and estrogen analogues, the many natural changes in hemostatic factors that occur in menopause, and the large number of prothrombotic factors of importance for thrombosis. Nevertheless, there is substantial evidence that conventional dose oral HT is associated with significant activation of coagulation. Importantly, it has recently been shown that even minor changes in clotting factors and coagulation inhibitors within normal ranges may account for such activation [210].

A strong correlation between an increase in levels of markers of activated coagulation and reduction in coagulation inhibitors has previously been demonstrated [69;118]. Even though we observed marked changes in coagulation inhibitors, regression analysis did not reveal any clear-cut relation between changes in a single coagulation inhibitor and changes in D-Dimer. This may be explained by the complex mechanism for activation of coagulation, i.e., by the combined effects of reduction of coagulation inhibitors and changes in clotting factors.

5.2.8 Proposed mechanism of inflammation

The discrepancies between the increased risk of arterial thrombosis and the beneficial findings in biochemical markers and observational studies are under debate. Numerous hypotheses have been suggested to explain these contradictory findings [92]. The observed reductions of levels of endothelial adhesion molecules in all treatment groups in the RET-study indicating an anti-inflammatory effect on the endothelial cells support the hypothesis of a beneficial influence of HT on vessel wall in younger women.

There is evidence that inflammation is involved in the development and progression of atherosclerosis, and that high level of CRP is an independent risk factor for atherosclerotic disease [55-57]. However, it is still unclear whether high levels of CRP directly contribute to pathogenesis of atherothrombosis [58], and whether the relationship between CRP and

inflammation is independent of other inflammatory markers. Reports showing no effects on CRP by transdermal HT [179;211] support the hypothesis that the changes in CRP are due to a direct effect on the hepatic cells. Despite this, we observed positive associations between changes in CRP and IL-6, VWF ag, and MCP-1 in a multiple regression analysis, showing that women with a large reduction in IL-6 had reduced increase in CRP. Similar pattern was seen for VWF ag and MCP-1. Apparently, these findings are conflicting. However, in our opinion they raise the hypothesis that the changes in CRP may be a result of both inflammatory stimulation mediated by IL-6 and general metabolic hepatic activation of estrogen [160]. These observations are in agreement with a recent report from the PEPI-trial, showing a positive correlation between changes in CRP and changes in IL-6 after treatment with oral combined HT [212]. In contrast there was a negative correlation between changes in CRP and changes in IL-6 in the estrogen monotherapy, suggesting that the effect of IL-6 on CRP production is related to the progestin-component of the treatment, and that the CRP changes after estrogen monotherapy are generated through a different pathway [212]. Moreover, reductions in IL-6 and VWF ag were significant predictors for reductions in CRP in the raloxifene group.

5.2.9 Link between coagulation and inflammation

Until recently, arterial thrombosis and VT have been considered unlinked disorders. There is now growing evidence for a possible association between arterial thrombosis and VT [213]. Recent reports suggest that these conditions share several risk factors and that their etiology are related to a combination of factors involved both in inflammation and thrombosis [213-215]. Several components of the vascular endothelium and coagulation factors have been suggested to be the link between inflammation and coagulation [52;216]. In a multiple regression analysis, we found that increases in CRP and IL-6 were significantly related to increase in D-dimer, both in the tibolone and the conventional-dose HT groups. Moreover, reductions in CRP and VWF ag were significant predictors for reductions in D-dimer in the raloxifene group.

5.3 Clinical implications

The many studies evaluating different doses and types of estrogen, different progestin formulation, and various routes of administration partly show conflicting results, which

render the clinical interpretations of these observations difficult. These divergent results might also be related to the age of the women, duration of treatment, coexistence of associated diseases, especially presence of atherothrombotic disease and other risk factors for hypercoagulability.

5.3.1. Biochemical markers and clinical importance

Several biochemical markers have been suggested to be associated with an increased risk of thrombosis [217]. First, it is important to emphasize that these markers are risk markers and not necessarily risk factors, involving causality. Nevertheless, in the EVTET study it was shown that the increased risk of VT was related to a marked activation of coagulation [118], and these changes were suggested to be attributed to reduced levels of coagulation inhibitors [69]. The mechanisms involved in thrombosis, i.e., the clinical and biological relevance of altered levels of various markers remains to be confirmed in RCTs. Additionally, the understanding of the mechanisms by which estrogen change the factors involved in thrombosis are incomplete (see Discussion of main findings).

Although the results from the RET-study indicate a differential effect of these four regimens on markers of vascular disease, the relationship between changes in these markers and risk of thrombosis remains unresolved. Our study was not designed to evaluate this issue; hence, our data do not provide any evidence for a relation between markers of vascular disease and thrombosis.

5.3.2 Relation to dose of hormone therapy

Only two epidemiological studies evaluating the risk of VT have examined dose relations [81;87]. Both these studies indicated an increased risk of VT with increasing dose of estrogen. However, the results did not reach statistical significance and must be interpreted with caution. Daly et al. included women using conventional-dose HT (0.625 mg CEE) in their low-dose group [87]. However, there is epidemiological evidence that lower oral doses may be associated with reduced risk of arterial thrombosis [91;93].

RCTs evaluating the risk VT of oral HT have almost uniformly utilized preparations containing either 0.625 mg CEE or 2 mg E2 [19-21]. RCTs evaluating the risk of VT on low-dose HT are lacking, except for the stroke-study of Viscoli et al. [88], where no excess risk of stroke, MI or VT was found. Moreover, two epidemiological studies have observed reduced

risk of arterial thrombosis on lower dose HT [91;93]. These findings fit well with the favorable effects that low-dose HT exert on inflammatory and endothelial markers [218], and the differential impact of the conventional and low-dose HT observed on hemostatic markers in the RET study. Furthermore, these findings indicate that low-dose HT has a more favorable risk-to-benefit profile than conventional-dose HT. Finally, it raises the question whether treatment with low-dose HT would yield different results regarding the risk of CVD in large RCTs. To address this question, RCTs utilizing low dose HT evaluating clinical end-points are warranted.

5.5.3 Impact of progestin

There is no data showing that progestin treatment alone used as OC or in intra uterine device (IUD) increases the risk of VT [219;220]. Although progestin-only pills seem to have minor impact on coagulation [221;222], it has been demonstrated in studies on different OC that different progestins attenuate the effects of estrogen on coagulation differently [44;221;223-226]. Moreover, there is evidence that OC containing different third-generation progestins increases the risk of VT [227;228]. Hence, the differential impact of the conventional and low-dose HT on most of the markers evaluated in the RET-study might be attributed both to the differential dose of estrogen and/or progestin utilized in the regimens.

5.3.4. Tibolone and raloxifene

The clinical implications of our findings on tibolone and raloxifene are not obvious. To our knowledge, no well-powered epidemiologic study nor clinical trial evaluating tibolone and the risk of VT has been performed [87]. However, a trial evaluating the effect of tibolone on the risk of vertebral fractures found that tibolone was associated with an increased risk of stroke [229]. Although our results showing that tibolone activates coagulation may indicate that tibolone has prothrombic properties, further clinical studies are required to conclude on the risk of VT with tibolone.

RCTs have shown that raloxifene is associated with an increased risk of VT [29-31], which may be comparable to that observed on conventional-dose HT. The risk of CVD associated with raloxifene are under debate [31;95;230;231]. In a large RCT, a subgroup of women at risk of CVD showed a reduction in coronary and cerebrovascular events when

assigned to raloxifene [95]. Since raloxifene in our study was not associated with activation of coagulation, other mechanisms for triggering thrombosis may be involved.

5.3.5 C-reactive protein - a predictor of venous thrombosis?

Significance of the proinflammatory effects of HT is controversial, and the clinical implications of an increase in CRP following HT are under debate. Based on the atherogenic properties of CRP [58] it has been suggested that the CRP increase induced by estrogen over many years may contribute to progression of atherosclerosis. It has been proposed that this partly can explain the observed increased risk of CVD by use of HT. However, in the WHI-study, the increased levels in CRP were not associated with increased risk of CHD [163]. In the EVTET-study, of women with a history of previous VT, the women who subsequently developed recurrence had a markedly higher increase in CRP at three months than those who did not (paper V). This finding must be interpreted with care because of the low number of cases (n=5). Nevertheless, our findings suggest that CRP might be a clinical predictor in recurrent thrombosis, as has been reported in an observational study [232].

5.3.6 Relation to route of administration

Transdermal application yields minimal effects on hemostatic and inflammatory markers [117;152;154;177-179;189] and is most probably not associated with increased risk of thrombosis [84;85]. Our findings, that oral HT is associated with a marked and rapid increase in CRP in women with high risk of thrombosis, whereas transdermal treatment is not, support the notion that transdermal therapy is safer. The increases in CRP in our studies were not accompanied by increases in IL-6, TNFα or other inflammatory markers, neither in healthy women (paper IV) nor in women with a high risk of thrombosis (paper V), indicating that this increase is due to hepatic activation and not to a general inflammatory response. Nevertheless, in the RET-study (paper IV) we observed a positive association between increase in CRP and IL-6, raising the hypothesis that the changes in CRP may be a result of a combined effect of both an effect of IL-6 and a direct effect on the liver cells (se Discussion of main findings).

Main Conclusions

- The various HT regimens and raloxifene exerted differential effects on markers of activated coagulation, coagulation factors, inhibitors and fibrinolytic factors, and markers of inflammation.
- Compared with the conventional-dose HT group, the low-dose HT group showed minor changes, suggesting a relation between dose of estrogen-progestin and effect on the various markers.
- Estrogen-progestin therapy induced an APC resistant phenotype, whereas tibolone and raloxifene only marginally altered the sensitivity to APC.
- Despite the marked changes in coagulation inhibitors, the regression analyses did not reveal any clear-cut relation between changes in a single coagulation inhibitor and changes in D-Dimer.
- An increase in average IL-6, TNF-α or other markers did not accompany the average increase in CRP, but women with large reductions in IL-6 had reduced increase in CRP.
- In women with high risk of thrombosis, oral HT containing E2, but not transdermal therapy was associated with a marked and rapid increase in CRP. The increase in CRP by oral treatment was not associated with increases of other inflammatory markers.

Future Perspectives

Contrary to earlier epidemiological studies suggesting protective effects of HT on CVD [90;91], recent RCT have demonstrated that HT in conventional doses induce a modest, but increased risk of CAD and VT [19-21;77-81]. It has also been shown that the increased risk of VT was related to a marked activation of coagulation [118] and these changes are suggested to be attributed to reduced levels of coagulation inhibitors [69]. Moreover, it has recently been shown that even minor changes in clotting factors and coagulation inhibitors within normal ranges may account for such activation [210]. These data support the hypothesis of a threshold by which estrogens may induce activation of coagulation, which might trigger thrombosis. Our results showing no impact of low-dose HT on markers of activated coagulation challenge the validity of the results from large RCTs utilizing conventional-dose HT, and it may be hypothesized that a lower-dose might have given lower risk of thrombosis. To address this question, RCTs utilizing low-dose HT evaluating clinical end-points are needed.

Unfortunately, no preparations with even lower doses of HT (ultralow) were available at the start of inclusion of the RET study. Recently, regimens with ultralow-dose of E2 and NETA have demonstrated its effectiveness on climacteric symptoms [233]. In order to find the regimen with the least potential harm, and further investigate the relationship between doses of estrogen/progestin, evaluating these regimens' effects on hemostatic markers are warranted.

The mechanism of HT-induced increased CVD is under investigation. Although no single coagulation factor has shown to be able to detect future thrombosis, multiple changes in hemostatic variables are measured and thrombogenic mechanisms have shown to be of importance. To investigate other possible candidates to explain the effects of HT on vascular disease a micro-array analysis in a subpopulation of the women in the RET-study has been performed. Hopefully, this analysis will contribute to elucidate the complex gene regulatory effects of ER-ligands and provide new insight on the mechanisms of thrombosis.

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